Activation of ERK1/2 by the human 5-HT_7 serotonin receptors

Dissertation for the *cand.pharm.* degree

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1 Abstract

Receptor tyrosine kinases (RTKs) activate the mitogen-activated protein (MAP) kinases called extracellular signal-regulated kinases 1 and 2 (ERK1/2) through a signaling cascade involving proteins such as Shc, Grb2, SOS, Ras, Raf-1 and MEK. Activation of ERK1/2 directs proliferation and differentiation in a wide array of cell types. G-protein-coupled receptors (GPCRs) of many types have been shown to activate the ERK cascade, but the complete mechanisms are yet to be elucidated. G_s-coupled receptors activate adenylyl cyclase (AC) leading to a rapid increase in cyclic AMP (cAMP), and the main targets for cAMP are protein kinase A (PKA) and the Exchange Protein directly Activated by cAMP (Epac) specific for the small G-protein Rap1. It has been proposed for the G_s-coupled β_2-adrenergic receptor that activation of ERK1/2 proceeds through Rap1 and B-Raf in a manner independent of Ras. However, for the human G_s-coupled serotonin receptors 5-$HT_{4(b)}$ and 5-$HT_{7(a)}$ it has been shown that ERK1/2 activation occurs independently of Rap1, relying instead on PKA and Ras.

In this project we have explored various signaling mechanisms originating from the human G_s-coupled 5-HT_7 serotonin receptors, with particular interest in the mechanisms of activation of ERK1/2.

We demonstrate the activation of ERK1/2 in HEK293 cells transfected with 5-$HT_{7(b)}$ and 5-$HT_{7(d)}$ receptors subsequent to stimulation with serotonin. For the 5-$HT_{7(b)}$ receptors, we show that the observed activation of ERK1/2 is dependent on cAMP and Ras, but not on Epac and Rap1. Furthermore, direct cytosolic calcium measurements have shown that treatment with serotonin leads to a rapid but transient elevation in intracellular calcium in HEK293 cells stably transfected with 5-$HT_{7(b)}$ receptors, and the observed phosphorylation of ERK1/2 is mediated, at least partly, through a calcium-dependent pathway. We show that HEK293 cells endogenously express the Ras specific, Ca^{2+}/calmodulin-dependent guanine nucleotide exchange factor Ras-GRF1. Ras-GRF1 becomes phosphorylated subsequent to 5-HT stimulation of 5-$HT_{7(b)}$ receptors, and it has previously been demonstrated that this phosphorylation enhances its towards Ras. This indicates that Ras-GRF1 has a role in the observed Ras-dependent activation of ERK1/2 mediated through 5-$HT_{7(b)}$ receptors.

2 Introduction

2.1 Cell Signaling

In a complex cellular arrangement like that of the human body, the ability of cell communication is of paramount importance. The cells exist and function exclusively for the benefit of the whole organism, and they must work together so as to direct and synchronize cellular functions. All of this relies on proper cellular signaling. Neuronal and endocrine/paracrine signaling are the two main strategies employed for this to be accomplished. These depend on and complement each other's role in the human cell system, and neither can be said to be more important than the other. Whereas neuronal signaling implies very direct and precise manipulation of target organs, (*i.e.* muscles, the adrenal medulla, the heart, the kidneys etc.) endocrine/paracrine signaling is much broader and slower, but may still affect the same tissues as neurons do.

Common for both of these methods is the utilization of chemical substances that bind to corresponding receptors, which then convey signals into the cells and direct cellular behavior. Neurons release neurotransmitters in the synaptic cleft, the 2 nm gap between itself and a target cell, and is consequently a very direct and clear-cut signal originating straight from the central or enteric nervous systems.

Other cells discharge mediators directly into the extracellular fluids surrounding them, and this constitutes a less discrete signal, but still just as important. Depending on the stability of these chemicals the signal can be classified as of endocrine or paracrine character. Hormones are examples of the former, and they are typically released by specialized glands into the blood stream from where they have access to the whole body through the circulation of the blood. Paracrine signals on the other hand are employed by all the cells in the body and function only in the near vicinity of the source, or even on the source itself (autocrine signaling). An instructive example of paracrine signals is found in the immune system with the plethora of cytokines these cells utilize to coordinate the body's immunological response to microbial infections.

An important aspect and common to all signaling mediators, is that they only affect cells displaying the proper receptors. Each cell in the human body produces a specific variety of receptors, and together they constitute an important part of the cell's identity, which is to say, its function and role in the cellular society. The same signaling molecule may also bind to receptors of different classes, so the combination of different signals reaching a cell, and the particular receptors it displays, determines its fate in the organism – proliferation, differentiation, movement, gene expression or apoptosis.

2.1.1 Cell receptors

Two main classes of receptors are present in the cell, those localized intracellularly and those embedded in the plasma membrane. Intracellular receptors are targets of lipophilic substances which cross the plasma membrane directly (*e.g.* steroid hormones and thyroid hormones). Such receptors are often gene regulatory proteins or in close proximity in signaling cascades to such, and their main function is to regulate the transcription of genes, either by induction or inhibition.

Plasma membrane receptors (table 2.1) on the other hand transmit signals from extracellular hydrophilic substances into the cell via an array of intracellular signaling molecules (also known as a signaling cascade). Plasma membrane receptors constitute a group of three main types, and these are: Enzyme-linked receptors, ligand gated ion channel and last but not least, G-protein coupled receptors (GPCRs). The latter is by far the most predominant and diverse. Common for all transmambrane receptors is an extracellular N-terminal domain to which the ligand binds, a transmembrane domain usually composed of α-helices and an intracellular C-terminal domain which conveys the signal to proteins inside the cell.

Table 2.1 Examples of plasma membrane receptors and their ligands

	Ligand	**Receptors**
Enzyme linked	Epithel growth factor (EGF)	EGFR
	Platelet derived growth factor (PDGF)	PDGFR
	Insulin-like growth factor (IGF)	IGFR
	Transforming Growth Factor β (TGF- β)	TGF-β receptor
	Atrial Natriuretic Peptide (ANP)	ANP receptor
Ion channel	Acetylcholine	Nicotinergic
	Serotonin (5-HT)	5-HT_3
	Gamma-aminobutyric acid (GABA)	GABAa receptor
	Glutamate	GluR channel, NMDA
	Glycine	NMDA
G protein-coupled	Acetylcholine (ACh)	Muscarinergic (M1-M5)
	Serotonin (5-HT)	5-HT receptors ($5\text{-HT}_{1, 2, 4\text{-}7}$)
	Catcholamines (NA)	Adrenergic (α1-2, β1-3)
	Histamine (H)	Histaminergic (H1-H2)
	Dopamine (DA)	Dopaminergic (D1-D5)

The enzyme-linked receptors consist of five main classes, each with considerable heterogeneity: Receptor tyrosine kinases are polypeptides with single transmembrane segments that form either hetero- or homo-dimers upon ligand binding. The receptors are activated by autophosphorylation on several tyrosine residues which subsequently may result in binding of proteins that direct signals into the cell. The other classes are receptor serine/threonine kinases (TGF-β receptor), receptor guanylyl cyclases (ANP receptor), tyrosine kinase associated receptors (cytokine receptors) and receptor-like tyrosine phosphatases (CD45).

The ligand gated ion channels usually consist of several transmembrane α-helices arranged in a way so that they form a pore with a specific diameter through which particular ions may pass (Na^+, K^+,Ca^{2+}, or Cl^-.) The extracellular side has binding sites for signaling molecules which after binding propagate a conformational change in the receptor which opens the pore, thus allowing the flow of ions from one side of the plasma membrane to the other. Ligand gated ion channels are typically situated in post synaptic membrane of the synapses between neurons or a target cell. The neurotransmitters released by the synapse operate as ligands for the receptor, and an excitatory or inhibitory signal is passed on to the postsynaptic cell, depending on what ion the receptor conducts.

2.2 G-Protein-Coupled Receptors (GPCRs)

G-Protein coupled receptors, also known as seven transmembrane segment receptors (7TMRs), heptahelical or serpentine receptors, is a group consisting of more than 2000 members, compromising >6% of the human genome. Over 100 subfamilies have been defined according to sequence homology, ligand structure and receptor function (1). GPCRs thus make up the largest and most diverse superfamily of proteins in the whole human body and since the cloning of the β_2-adrenergic receptor (β_2-AR) for catecholamines in 1986 (2), extensive experimental work has been elicited to uncover the variety of their functions.

Ligands for the GPCR group include many small chemical molecules, nucleotides, nucleosides, peptides, lipids, proteins, hormones, neurotransmitters, pheromones as well as retinal, the light activated ligand of rhodopsin. The importance of GPCRs can be envisioned by the fact that more than 60% of the drugs utilized today have these receptors as their targets, either directly or indirectly. There is still much work to be done before we understand completely the physiological, biochemical, pathological and pharmacological backgrounds of these complex signaling networks.

2.2.1 The basic structure of GPCRs

On the level of primary and secondary structure, much information is available since many GPCRs have been sequenced and cloned. Overall, these receptors do not share much amino acid homology and the only structural feature common to all GPCRs is the presence of the seven transmembrane (TM) spanning α-helical segments connected by alternating intracellular and extracellular loops, with the amino terminus located on the extracellular side and the carboxyl terminus on the intracellular side (fig. 2.1). The transmembrane segments cluster in the membrane and play a vital part in ligand binding and transduction of the signal into the cell. The α-helices are termed transmembrane 1 (TM1) through transmembrane 7 (TM7) starting at the N-terminal end. Three extracellular and three intracellular loops are formed between the TM segments. In some GPCRs, a fourth intracellular loop is formed when a part of the C-terminal tail is palmitoylated at a cysteine

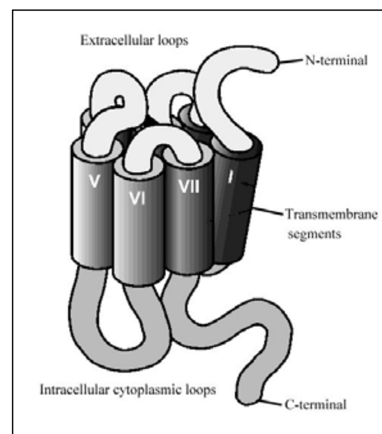


Figure 2.1 The basic structure of GPCRs.

residue and anchored to the plasma membrane. The extracellular loops and the N-terminus play a role in ligand binding whereas the intracellular loops and the C-terminus are involved in interaction with G proteins (see chapter 2.2.3). Each of the seven α-helices is composed of 20-27 hydrophobic amino acids. The N-terminal and C-terminal segments, as well as the intracellular and extracellular loops vary considerably in length (5-600 amino acids) indicating a diversity of functions.

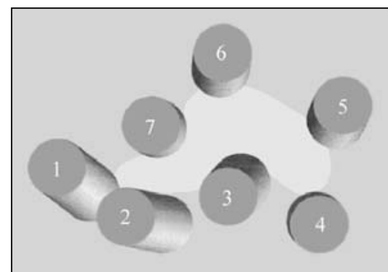


Figure 2.2 The alignment of transmembrane segments in the plasma membrane seen from the extracellular side.

Based on marginal sequence similarities, three major families and several subfamilies of GPCRs have been defined (table 2.2). Sequences within each family generally share over 25% identity in the transmembrane core regions. The family A receptors are by far the best studied, and share some conserved key residues thought important for structural and functional integrity (The DRY (Asp-Arg-Tyr) motif in the third intracellular loop being the prime example). For the family B receptors, the most prominent feature is a large (~100 amino acids) extracellular N-terminus, containing several cysteines, probably involved in forming a network of disulphide bridges. Family C receptors are characterized by very large N-termini (500-600 amino acids) thought to play a role for agonist binding.

Table 2.2 Various G protein-coupled receptor families

Family		***Receptors***
A	Rhodopsin/ β_2-AR family	Receptors for adrenaline, serotonin, dopamine, acetylcholine, histamine, endothelin, bradykinin, adenosine, cannabinoids, eicosanoids, chemokines, opioides, vasopressin, oxytocin, and many more.
B	Glucagon receptor family	~25 members. Peptide hormones and neuropeptides (Glucagon, secretin, VIP, calcitonin, GHRH, CRH and PTH).
C	Metabotropic neurotransmitter receptors	Glutamate, GABA, Ca^{2+}, pheromone and taste receptors.

2.2.2 Function of GPCRs

2.2.2.1 Basic agonist binding and signal transduction theory

G-protein-coupled receptor signaling is made up of three distinct molecular components: discriminators (receptors), transducers (G-proteins), and amplifiers (effectors). Simply stated, the discriminator, the GPCR, is stimulated by its specific extracellular ligand. Agonist binding occurs by several mechanisms, for example: binding within a hydrophobic ligand pocket formed by the seven TM spanning domains (family A), binding to the extracellular loop domains and the amino-terminal tail of the receptor (family B) or binding to a stretch of amino acids within the long extracellular amino-terminal tail (family C).

Agonist binding leads to a conformational change in the receptor, allowing it to recruit specific heterotrimeric G-proteins – the transducers. They in turn convey the signal to

intracellular amplifiers as described in the next chapter. The current theory explaining GPCR activity suggests that the GPCRs can spontaneously isomerize between inactive and activated receptor conformations leading to the non-agonist induced activation of G-proteins. Consequently, GPCRs exhibit some degree of spontaneous constitutive activity in the absence of agonist stimulation. Receptor activation by agonists merely promotes the stabilization of the activated receptor conformation, thereby potentiating interaction with G proteins (3).

Inverse agonists are drugs that do not stimulate receptor activation but rather selectively recognize or stabilize the inactive receptor conformation and thereby reduce the spontaneous or intrinsic activity of GPCRs. Full antagonists do not preferentially recognize or stabilize either the inactive or active receptor conformation but competes with agonists for receptor binding. Thus, the treatment of cells with full antagonists will result in no net change in receptor activity in the absence of agonists; rather, the antagonists will block the activity of both customary agonists and inverse agonists if they are present. Inverse agonists on the other hand, have the ability to abolish the receptor's intrinsic activity.

2.2.3 G-proteins

G-proteins are so-called because they bind the guanine nucleotides GDP and GTP. They are activated by the exchange of GDP for GTP, and subsequently inactivated by the hydrolysis of GTP to GDP (4).

2.2.3.1 Small G-proteins

Small monomeric G-proteins rely on external guanine nucleotide exchange factors (GEFs) for their activation, and GTPase activating proteins (GAPs) for deactivation. An instructional example is the human homologues of Harvey or Kirsten Rat Sarcoma virus oncogenes, the p21 Ras family of GTP-binding proteins. This family consists of three members, Ha-Ras (also known as H-Ras), Ki-Ras (K-Ras) and N-Ras. Ras is activated by guanine nucleotide exchange factors carrying the CDC25 homology domain. To date, the GEFs mSOS, Ras-GRF1/2 CalDAG-GEFII (RasGRP) and CalDAG-GEFIII (RasGRP3), as well as PDZ-GEFs have been found to contain such a domain, and can thus active Ras (5,6). CalDAG-GEFI and certain PDZ-GEFs activate the Ras-related proteins Rap1 and Rap2 which are also small monomeric G proteins.

Ras displays a weak intrinsic GTPase activity that mediates the hydrolysis of bound GTP to GDP and thereby inactivation of Ras. However, the inactivation of Ras is enhanced by GAPs such as GAP1^{m}, NF-1 and p120 GAP (6). Ras is a major component of the mitogen activated protein kinase cascade, involved in growth factor signaling as well as GPCR signaling. Constitutively active mutants of Ras can give rise to uncontrolled growth and act as potent oncogenes. The role of Ras in MAPK cascades will be discussed more thoroughly in chapter 2.3. The exchange factor Ras-GRF1 is presented in detail in chapter 2.3.1.4.

Other examples of small monomeric G-proteins in the Ras family of GTP-binding proteins are Rho, Rac and Cdc42. They regulate various physiological responses through actin cytoskeleton rearrangements (7).

2.2.3.2 Large polymeric G-proteins

Polymeric G-proteins consists of at least one subunit that shows intrinsic GTPase activity, which thereby constitutively deactivates the protein by hydrolysing GTP.

Heterotrimeric G-proteins are made of three different subunits, α, β and γ, and are responsible for conveying the primary signal from the GPCR to an intracellular amplifier. The α-subunit is involved in receptor-effector coupling and is bound to the plasma membrane by a myristoylated and palmitoylated N-terminus. It exerts GTPase enzymatic activity and binds either a molecule of GTP (activated state) or its hydrolysis product GDP (inactivated state). The artificial GTP analogue GTPγS cannot be hydrolyzed and may thus be used to activate the α-subunits for very long periods under experimental conditions.

The β- and γ-subunits are tightly associated and do not separate *in vivo*. The two subunits are commonly referred to as the Gβγ-dimer, and are attached to the plasma membrane by a geranylgeranyl group at the C-terminus of the γ-subunit. The Gβγ-subunit can play several roles in intracellular signaling, for example, facilitation of receptor phosphorylation and desensitization, activation of K^+ channels, modulation of Gα-activation, activation of PLA_2 and PLC, activation of PI3K and regulation of calmodulin. It is however, the Gα-subunit which is generally thought of as the main effector of signal transmission. The Gα-subunits are divided into four major classes, $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$ (see tables 2.3 and 2.4), with several subtypes adding to the complexity.

Table 2.3 The four major classes of the Gα-subunit.

	Immediate targets	**Cellular effects**
$G\alpha_s$	Activation of adenylyl cyclase (AC)	AC catalyzes the formation of cAMP from ATP. cAMP activates PKA and Epac.
$G\alpha_i$	Inhibition of AC (as well as activation of inwardly rectifying potassium channels.)	Inhibition of cAMP formation.
$G\alpha_q$	Activation of phospholipase Cβ (PLCβ)	PLCβ cleaves membrane phosphotidylinositol-4, 5-bisphosphate (PIP2) to release two second messengers: diacylglycerol (DAG) and inositol-(1, 4, 5)-trisphosphate (IP_3). IP_3 causes release of Ca^{2+} from intracellular stores, which together with DAG activates PKC. Ca^{2+} is in it self a ubiquitous second messenger and can modulate the activity of many proteins.
$G\alpha_{12}$	Activation of Rho GEFs	Activation of Ras (directly via GEFs) and the Jun N-terminal kinase signaling module and Na^+/H^+ exchangers.

The $G\alpha_s$-subunit is the target of the cholera toxin (CTx) liberated by *Vibrio cholerae*. CTx catalyzes the transfer of ADP-ribose to $G\alpha_s$ which inhibits its intrinsic GTPase activity and thus makes $G\alpha_s$ constitutively active. This causes persistent activation of adenylyl cyclase. Associated with the epithelial lining of the intestine, this causes a massive loss of salts and water from the cells, a severe diarrhea, the predominant symptom of a *V.cholerae* infection.

The $G\alpha_i$-subunit on the other hand, is the target of pertussis toxin (PTx) which is produced by *B.pertussis*. PTx ADP-ribosylates $G\alpha_i$, and this prevents $G\alpha_i$ from interacting

with its activated receptor, causing it to be retained in its GDP bound state. $G\alpha_i$ thereby sequesters Gβγ-subunits, and fails to inhibit AC with a resulting increase in intracellular cAMP. Both CTx and PTx are employed in various ways as experimental tools to investigate signal transduction pathways.

There is considerable diversity in the G-protein subunit gene family. Cloning and sequencing of cDNAs has defined not only the four main classes of α-subunits, but also at least 16 isotypes within this gene family. The β- and γ-subunits also exist in several isoforms. At least 6 of the former and 12 of the latter have been identified, and some of the subunits have splice variants or are differentially modified, contributing to the total diversity. Taken together there are more than 1000 theoretically possible combinations of heterotrimeric G-proteins, and it is thought that expression of different isotypes within a G-protein confer a type of subtle long term regulation of signal transduction in a cell, either by dampening or sensitization. It is also becoming clear that individual GPCRs on the cell surface recognize specific combinations of heterotrimeric G-proteins, which implicates that two receptor isotypes binding the same ligand may produce different effects intracellularly (8).

As described before, the binding of ligand to the receptor results in a conformational change in the receptor's intracellular regions, allowing interaction with specific G- proteins. This interaction in turn causes a conformational change in the G-protein which facilitates release of GDP and binding of GTP, leading to dissociation of the Gα- and Gβγ-subunits. The separated subunits can then bind to and regulate various intracellular effectors.

Table 2.4 Various G-protein coupled receptors (GPCRs), their ligands and G-proteins

Receptor	Ligand	G-Protein
β_1-AR, β_2-AR, β_3-AR	Catecholamines (NA)	$\mathbf{G_s}$
D_1, D_5	Dopamine (DA)	
5-$HT_{4(a)}$, 5-$HT_{4(b)}$, 5-HT_6, 5-$HT_{7(a)}$, 5-$HT_{7(b)}$, 5-$HT_{7(d)}$	Serotonin (5-HT)	
H_2	Histamine	
A_{2A}, A_{2B}	Adenosine	
M_1, M_3, M_5	Acetylcholine	$\mathbf{G_q}$
α_{1A}-AR, α_{1B}-AR, α_{1C}-AR, α_{1D}-AR	Catcholamines (NA)	
5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}	Serotonin (5-HT)	
H_1	Histamine	
ET_A, ET_B	Endothelin	
M_2, M_4	Acetylcholine	$\mathbf{G_{i/o}}$
α_{2A}-AR, α_{2B}-AR, α_{2C}-AR	Catecholamines (NA)	
D_2, D_3, D_4	Dopamine (DA)	
5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}	Serotonin (5-HT)	
A_1, A_3	Adenosine	

2.2.4 Adenylyl cyclase and cyclic AMP

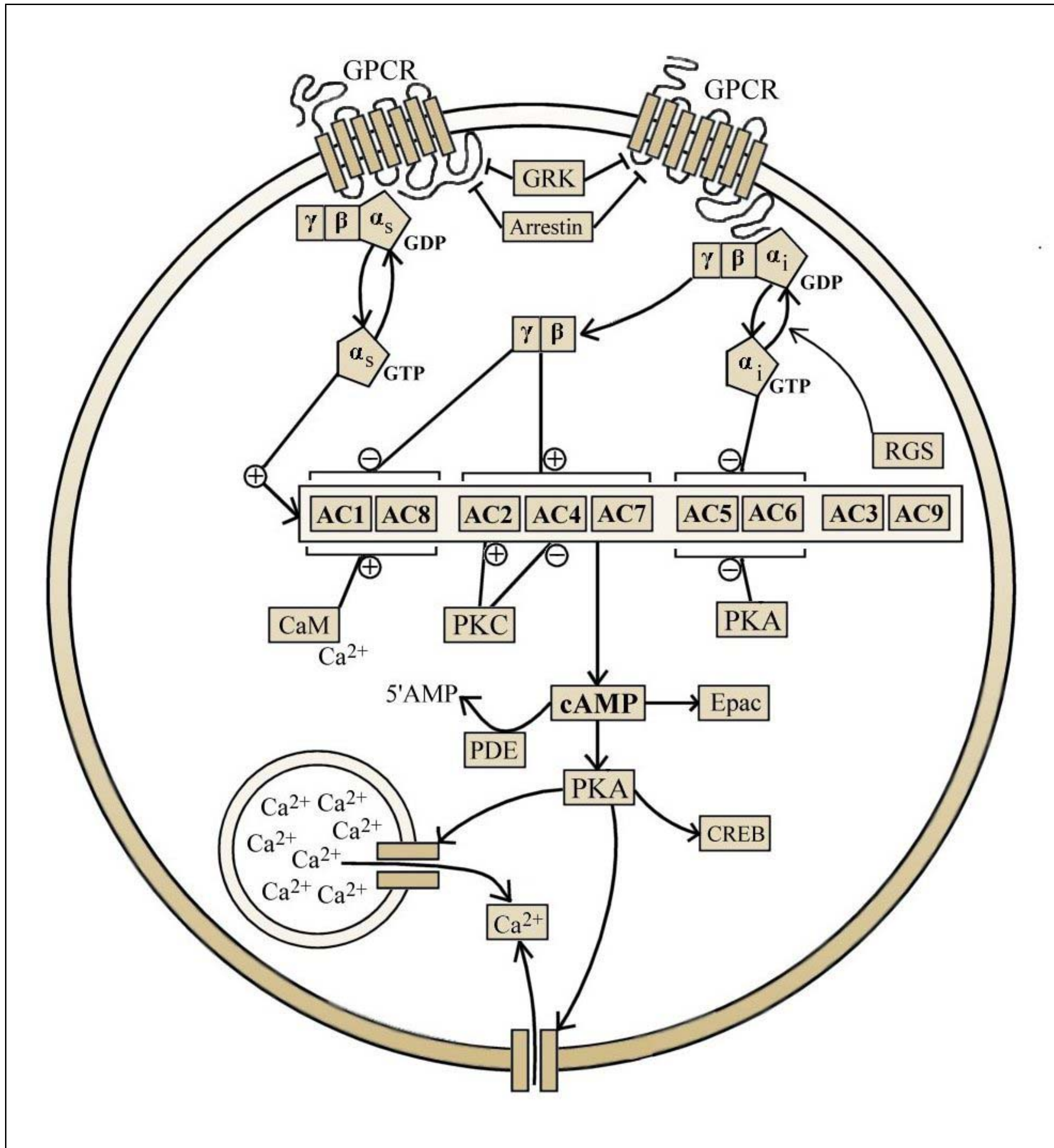


Figure 2.3 Activation and inhibition of adenylyl cylcase by several mechanisms.
GRK: G-protein coupled receptor kinase, AC: Adenylyl cyclase, CaM: Calmodulin, Epac: Exchange factor directly activated by cAMP. RGS: Regulator of G-protein signaling, CREB: cAMP response element-binding protein, PDE: Phosphodiesterase.

The Gα_s-subunit activates adenylyl cyclase which catalyzes the formation of cAMP from ATP. The binding of an extracellular ligand to a G_s-coupled receptor may cause a more than 20-fold increase in intracellular levels of cAMP within seconds (9). The rapid production of cAMP is accompanied by the equally rapid breakdown to AMP by specific phosphodiesterases (PDEs). There are eleven families of PDEs (PDE1 – PDE11) with

several isotypes within each family. They differ in tissue and cellular distribution as well as regulation, and play a major role in controlling the response from cAMP, discussed in chapter 2.2.6.

There are at least nine different isoforms of adenylyl cyclase (designated AC1 through AC9). They differ considerably in regulatory properties and expression levels in various tissues. The AC4, AC7 and AC9 isoforms are found in most cells, whereas AC1 and AC8 are restricted to neuronal tissues and AC5 is found mainly in the heart and brain (10).

Adenylyl cyclases are integral membrane proteins that consist of two bundles of six transmembrane segments. Two catalytic domains extend as loops into the cytoplasm and comprise the enzymatic actions of the protein.

Regulation of AC is not a simple matter of turning on and off by $G\alpha_s$ and $G\alpha_i$, respectively. $G\beta\gamma$-dimers, PKC, PKA, calmodulin dependent kinase II and calcium/calmodulin are all implicated in AC regulation, either by activation or inhibition (11). Ultrastructural labelling has demonstrated a close spatial association of ACs with sites of calcium entry in many cell types. It thus appears that there is tight integration between cAMP and calcium, the cell's two major second massagers (12). The specific consequences of these cooperative networks with accompanying complex regulatory mechanisms remain to be elucidated. Integration of inputs from several signaling pathways seems to play a crucial role in cellular control (13).

Elevation of cAMP is primarily associated with the activation of Protein Kinase A, but cAMP is also known to activate Epac and some ion channels (14).

The classic effect of cAMP mediated activation of PKA is common to all cells in which AC is present, but the final cellular outcomes vary considerably between different tissues and depend on proteins further down in the cascade, as summarized in table 2.5. This is true for all the G-protein signaling cascades, and in such a perspective, the GPCRs serve as general templates for the delivery of signals to cells.

Table 2.5 Some physiological effects of elevated intracellular cAMP

Ligand	**Tissue**	**Effect**
Adrenaline/Noradrenaline	Muscle	Glycogen breakdown
Adrenaline/Noradrenaline	Heart muscle	Increased inotropy and chronotropy
Adrenocorticotrophic hormone (ACTH)	Adrenal cortex	Cortisol secretion
TSH	Thyroid gland	Thyroid hormone synthesis and secretion
Glucagon	Liver	Glycogen breakdown
Adrenaline, ACTH, glucagon, TSH	Adipose tissue	Triglyceride breakdown

2.2.5 Protein Kinase A

The cAMP dependent serine/threonine kinase, PKA, is the main target of cAMP and has been implicated in regulation of a wide range of cellular processes, including transcription, metabolism, cell cycle progression and apoptosis.

Inactive PKA is a complex consisting of two regulatory (R-) subunits and two catalytic (C-) subunits, R_2C_2. Each R-unit must bind two molecules of cAMP (thus a total of four for the whole PKA complex) before activation can come to pass. The C-units are then released from the protein complex, thereby exposing their catalytic kinase domains. There is an excess of R subunits in the cytoplasm, and therefore when cAMP levels drop, the catalytic subunits are rapidly inactivated by the binding of R-subunits and reassembly of the protein complex.

There are two forms of regulatory subunits present in eukaryotic cells, RI and RII, and each exists in two isoforms. PKA is classified as type I or type II depending on the associated R subunit. The former is predominantly found in the cytosol whereas the latter associates with cellular structures and organelles. Cellular localization is proving to be of considerable significance for PKA. Type II PKA is not a free floating enzyme, but is anchored to precise locations by so-called A Kinase Anchoring Proteins (AKAPs). Type I AKAPs have also been characterized, and the distinct localization of different PKAs keeps them in range of specific targets and prevents random and indiscriminate phosphorylation (15).

The catalytic subunits of PKA function independently and exist in three isoforms (16). Each C-subunit consists of two domains, a small domain with several β sheet structures and a larger domain containing several α helices (17). The binding sites for the substrate and ATP are located in the catalytic cleft between the domains, and when ATP and the substrate bind, the two lobes rotate so that the terminal phosphate group of the ATP and the target amino acid of the substrate move into the correct positions for the catalytic reaction to take place, which is the transfer of one phosphate group from ATP to a serine or threonine residue on a protein (17).

One target of phosphorylation by PKA is the cAMP Response Element Binding Protein (CREB) found in the nucleus of most eukaryotic cells. Phosphorylated CREB (pCREB) increases activation of transcription, and pCREB is sometimes used as an indicator of PKA activity. This, however, can produce false positive results since CREB is promiscuously phosphorylated, for example by ERK1/2 (18).

PKA phosphorylation may entail inactivation as well as activation. Examples of the former kind are: myosin light chain kinase and Raf-1 (19), whereas examples of the latter are Src (20), Rap1 (21) and Ras-GRF1 (22).

2.2.6 Regulation of GPCR effector cascades

For signal transduction to be effective it must be under tight regulatory control. Turning off a signal is equally important as turning it on, and there are many systems in place to regulate the signals from GPCRs. Feedback inhibition is a common cellular strategy, and is often executed by the key protein in a signaling cascade. This ensures that deactivation follows promptly after activation, in fact, they are often two sides of the same effector system.

For example: Signaling through G_s-coupled receptors may be inhibited by direct phosphorylation and inhibition of the receptor mediated by PKA (see chapter 2.2.6.1). PKA may also phosphorylate some adenylyl cyclase isoforms and thereby inhibit the formation of cAMP. Finally, certain phosphodiesterases are phosphorylated by PKA and this increases their effect, causing an increased breakdown of cAMP. Taken together, PKA helps diminish the sources from which its own activation originates, and thereby shuts the signal off. In this respect, PKA may be equally important as a propagator of an intracellular signal, as it is for terminating the very same signal.

The hydrolysis of GTP from G proteins is facilitated by the presence of specific modulators called RGS proteins (Regulator of G protein Signaling). They act as α-subunit-specific GTPase activators and thus inactivate G proteins. A total of 25 RGS proteins are encoded by the human genome, and they all interact with a particular set of G proteins to shut off their responses. AGS (Activators of G protein signaling) on the other hand, can activate G protein in the absence of GPCRs (23).

2.2.6.1 Heterologous and homologous GPCR desensitization

Agonist independent phosphorylation of GPCRs is termed heterologous receptor desensitization, as opposed to homologous desensitization which only affects receptors activated by agonists.

Inactivation of the G protein-coupled receptors is accomplished by several mechanisms, and one proceeds through PKA or PKC. These kinases are key proteins in their respective signaling cascades (i.e. the G_s and G_q initiated cascades) and they have the ability to phosphorylate GPCRs. This inhibits the ability of the receptor to associate with G-proteins, and disrupts the signaling cascade. Both agonist activated receptors and receptors that have not been exposed to agonist may be inhibited in this fashion. A consequence of the latter is that activation of PKA from one G_s-coupled receptor may inhibit a variety of other GPCRs (i.e. heterologous desensitization).

Another mechanism for PKA mediated desensitization has been proposed recently. In the case of the β_2-AR, phosphorylation of the receptor has been suggested to switch coupling from G_s to G_i (24). This has also been implicated in activation of growth promoting pathways as described in chapter 2.3.2.3, but the hypothesis of G-protein switching is controversial and is not generally accepted (25).

G-protein coupled receptor kinases (GRKs) specifically phosphorylate and desesensitize agonist activated GPCRs and are thus obligate mediators of homologous desensitization. Seven distinct isoforms of GRKs exist: GRK1 through GRK7. GRKs are cytosolic proteins which are recruited to the plasma membrane in response to GPCR activation. The β-AR kinases (βARK1 (GRK2) and βARK2 (GRK3)) are targeted to the plasma membrane by

the association of the C-terminal domain of the kinase with the βγ-subunit of the activated G protein. GRKs phosphorylate residues within the third intracellular loop and carboxyl-terminal domain of GPCRs. This in itself contributes to inactivation of the receptors, but it also increases the receptor's affinity to arrestins (*e.g.* β-arrestin) which uncouples the receptor sterically from G-proteins, and may target the receptor for endocytosis (see below).

2.2.6.2 Endocytosis of GPCRs

The internalization of agonist-activated receptors serves to reduce the number of cell surface receptors that are available for ligand binding. The mechanisms involved in targeting GPCRs for internalization are the same as those involved in receptor desensitization. GPCR endocytosis usually requires GRK phosphorylation followed by β-arrestin binding. β-arrestins interact with clathrin, targeting agonist-activated GPCRs to clathrin coated pits. These pits invaginate and form vesicular structures that pinch off from the cell surface in a dynamin dependent manner. The vesicles then carry their receptor cargo to endosomal membrane compartments where several outcomes may follow (26). In the case of receptors like the β_1-AR and the endothelin A receptor, internalized GPCRs are dephosphorylated and recycled back to the plasma membrane surface as fully functional receptors. On the other hand, some GPCRs (*e.g.* the endothelin B receptor, and the β_2-AR) are usually targeted to lysosomes for degradation (27,28). Recycled receptors can thus mediate persistent responses to agonist, whereas degraded receptors only mediate transient responses since the availability of receptors diminishes. Endocytosis of GPCRs is not only implicated in terminating signaling, but also, paradoxally, in the triggering of completely new and rather unconventional signals. For example mitogen activated protein (MAP) cascades which are normally linked to growth factor receptors. These concepts and more are the subject of the following chapters.

2.3 The Mitogen Activated Protein Kinase Cascade

MAPK families play an important role in complex cellular programs like proliferation, differentiation, development, transformation, and apoptosis. At least three MAPK families have been characterized: The extracellular signal-regulated kinases (ERK), Jun kinases (JNK/SAPK, c-Jun amino-terminal kinase) and p38 MAPK. These signaling networks regulate the cell cycle machinery and other cell proliferation related proteins.

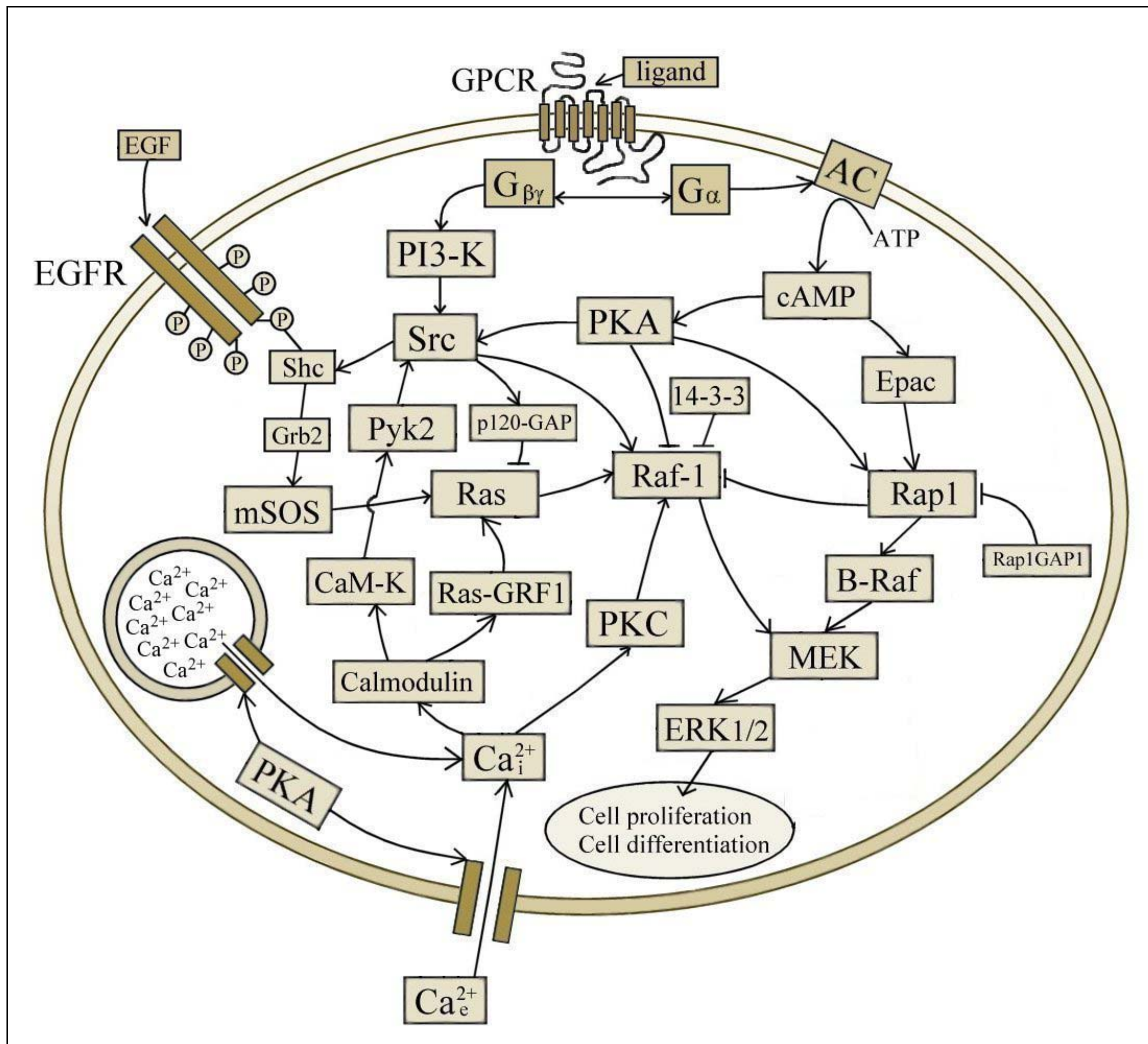


Figure 2.4 Many roads lead to Rome. A schematic review of ERK1/2 activation by RTKs and the the many proposed mechanisms of ERK1/2 activation by GPCRs. For further details, consult with the following chapters.

2.3.1 Receptor tyrosine kinases activate the MAPK cascades

Receptor tyrosine kinases (RTKs) are found in all multicellular eukaryotic organisms, and according to classic signal transduction theories, growth inducing signals are conveyed primarily by these receptors. The signaling cascade mediating activation of MAPKs via RTK is portrayed roughly in the upper left corner of figure 2.4. Other MAPK cascades exist apart from the classical Ras/Raf/MEK/ERK pathway. Of particular importance are the JNK and the p38 cascades. Both are activated by stress, cytokines and growth factors and they regulate proliferation differentiation and apoptosis.

2.3.1.1 The Src kinase family

The Src family of non-receptor tyrosine kinases plays an important part in the activation of MAPK cascades by RTKs, both structurally and functionally. The Src family consists of nine members in mammals: Src, Yes, Fgr, Yrk, Fyn, Lyn, Hck, Lck and Blk, with various biochemical characteristics and an array of cellular targets for phosphorylation (29).

Src kinases contain so-called SH (Src homology) domains, *i.e.* SH1, SH2, SH3 and SH4. Put shortly, the SH1 domain constitutes the catalytic domain of the protein (kinase activity), the SH2 domain binds to phosphorylated tyrosine residues, the SH3 domain binds to proline rich regions and the SH4 domain is important for membrane localization.

In addition to their SH2 and SH3 domains, Src kinases also contain the proline rich regions to which SH3 domains bind (SH3 *domain binding motif*) as well as a SH2 *domain binding motif* (a phosphorylated tyrosine residue) on its C-terminal tail. Thus, Src can bind to itself, and consequently downregulate its own activity by forming a closed, inactive conformation (30). The loss of these bindings as a result of mutations can lead to a constitutively active Src molecule and increased growth. Such mutants of Src act as oncogenes. The viral cancer-causing counterpart of Src from the Rous sarcoma virus (v-Src) exists only in the active conformation.

Src phosphorylates and activates Raf-1 and Shc, promoting ERK1/2 activation (20) (see chapter 2.3.1.2). Furthermore, Src phosphorylates p120-GAP and thereby *inhibits* its Ras-GAP activity, thus also promoting ERK1/2 activation (31). Src itself can be activated by PKA phosphorylation and inactivated by Csk phosphorylation on distinct sites.

2.3.1.2 The RTK/mSOS/Ras/Raf/MEK/ERK cascade

When RTKs are activated, various SH2 domain containing adaptor proteins can bind to the receptor's intracellular autophosphorylated tyrosine residues. Examples are, apart from the Src kinases, PLCγ, Shc, Crk, Gab-1 and Grb2.

Shc serves as an adaptor for SH2 domain containing proteins as it also has an SH2 domain binding motif. Grb2 may thus bind directly to the receptor, or to a phosphorylated Shc. Many proteins contain both SH3 and SH2 domains. Crk is one example, and may bind to phosphorylated RTKs through its SH2 domain and to C3G (a Rap GEF) through its SH3 domain. Another example is Src, which may bind both to RTKs and to the p85 subunit of PI3K, a protein which has proliferative and antiapoptotic effects through many mechanisms, the most important being activation of Akt kinase (PKB). PI3K may also bind to activated RTKs through the adaptor protein Gab-1. A third example is Grb2, to which the Ras GEF mSOS may bind.

When bound to Grb2, mSOS is in a position to activate the membrane anchored small monomeric G-protein Ras. Activated Ras may bind to the N-terminal Ras binding domain of Raf-1 (RafRBD), thereby targeting the latter protein to the plasma membrane. The Raf-family comprises three members in mammals: A-Raf, Raf-1 (c-Raf) and B-Raf. The activity of Raf-1 is regulated through a complex set of mechanisms involving protein-protein interactions, phosphorylation and dephosphorylation on multiple residues.

Raf-1 is functionally inhibited when bound to the protein dimer 14-3-3, and PKA phosphorylation on Raf-1 potentiates this binding (19). Src phosphorylation on the other hand, relieves intrinsic autoinhibition of Raf-1 (20).

When Raf-1 binds to Ras, the inhibitory 14-3-3 protein is prevented from binding, and as a result, Raf-1 becomes activated. Raf-1 is in turn a protein kinase (known as a MAP kinase kinase kinase, MKKK) which mediates phosphorylation and activation of the MAP kinase kinases MEK1 and MEK2 (MAP & ERK Kinases) on the serine residues 218 and 222. Finally, MEK1 and MEK2 dually phosphorylate and activate ERK1 and ERK2 (Extracellular Signal-Regulated Kinases, or MAP kinases p44 and p42) and this completes the sequence.

2.3.1.3 Extracellular signal-regulated kinases

Five subtypes of these MAP kinases exist, designated ERK1 – ERK5, ERK 1 and ERK2 (ERK1/2) are the best studied. Dual phosphorylation on Thr202 and Tyr204 on ERK1 and Thr183 and Tyr185 on ERK2 by MEKs is necessary for the full activation of these MAP kinases. Activated ERK1/2 have targets in the cytosol, but also translocate into the nucleus and activate transcription factors, changing gene expression to promote growth, differentiation or mitosis. ERK1/2 play a pivotal role in the control of cell cycle progression.

2.3.1.4 Ras-GRF1

The Ras specific exchange factor Ras-GRF1 ($CDC25^{Mm}$) is localized to the plasma membrane through an amino-terminal Pleckstrin homology (PH) domain (32). How this targeting is accomplished is somewhat unclear, but it may involve binding to either membrane phospholipids (*e.g.* phosphoinositides) or Gβγ subunits.

Ras-GRF1 is activated by calcium and the release of Gβγ-subunits from heterotrimeric G proteins (33). Ca^{2+} dependent activation is mediated through binding of calmodulin to an IQ motif on Ras-GRF1 (34). Furthermore, PKA mediated phosphorylation on Ser898 in the human sequence (which corresponds to Ser916 in the mouse sequence) is required for full activation (22). Ras-GRF1 is also a substrate for CaM Kinase II (35), but the cellular significance of this is unknown.

Ras-GRF1 has also been shown to exhibit Rac GEF activity, mediated by Src phosphorylation, but this phosphorylation does not influence Ras GEF activity (36). Rac is known to activate the JNK cascade. On the other hand, Lck, another member of the Src family kinases, is able to stimulate the GEF activity of Ras-GRF1 on H-Ras, thus promoting activation of ERK1/2 (31).

Ras-GRF1 has been reported to be found mainly in brain neurons (37), and it is highly expressed in the rat brain compared with the mSOS (22). It is thus thought to play a

significant role in Ras activation in the CNS. Ras-GRF1 has also been found in the lung and pancreas, and several tumor cell lines (38,39). However, its full role in ERK1/2 activation resulting from GPCR signaling is yet to be determined.

2.3.2 Activation of ERK1/2 by GPCRs

Since the discovery that GPCR signaling cascades can activate the pathways originally thought of as exclusive for RTKs, the prospects of crosstalk and transactivation has been a field of intense research. Current data suggests that there is no single dominant pathway for ERK1/2 activation via GPCRs, not even from a single class of GPCRs. It seems that several pathways work in cooperation or opposition, and produce a mutable system which is dependent on the specific complement of GPCRs present and the available machinery in the particular cell. Transactivation of RTKs, or crosstalk with the typical MAPK proteins, seem to be important, but exactly how this is accomplished is still shrouded with uncertainty. This uncertainty has arisen because mechanisms operating in a particular cell type for specific GPCRs may not operate correspondingly in another model system (40). For a brief summary of current theories, confer with figure 2.4. It seems there are as many mechanisms as there are scientists exploring them. The following chapters consider more thoroughly how different classes of GPCRs may activate ERK1/2.

2.3.2.1 Activation of ERK1/2 through G_q-coupled receptors

G_q-coupled receptors activate ERK1/2 in a process involving PLC, IP_3, Ca^{2+} and PKC. PKC can phosphorylate and activate Raf-1 directly, which in itself is sufficient to activate ERK1/2.

In addition, Ca^{2+} can activate several CaM dependent kinases, which may phosphorylate Pyk2 (proline-rich tyrosine kinase 2). Phosphorylated Pyk2 acts as a docking site which can bind and activate the cytoplasmic kinase Src (41) which subsequently may induce activation of Raf-1 and Shc. Shc binding to Src can recruit Grb2 to Pyk2 with the resulting activation of the ERK1/2 cascade through mSOS and Ras with further activation of Raf-1.

The increased intracellular levels of Ca^{2+} may also activate calcium dependent GEFs specific for Ras as described in chapter 2.2.3.1. This may also contribute to the final activation of ERK1/2 mediated by G_q-coupled receptors.

2.3.2.2 Activation of ERK1/2 through G_i-coupled receptors

G_i-coupled receptors are proposed to activate ERK1/2 through the $G\beta\gamma$-subunit. The use of PTx which effectively inactivates $G\alpha_i$ and sequesters $G\beta\gamma$-subunits (as discussed under chapter (2.2.3.2), has shown that the $G\beta\gamma$-subunit is necessary for the activation of ERK1/2 (42). Consistent with this, overexpression of $G\beta\gamma$-subunits is sufficient to activate ERK1/2 through a mechanism dependent on PI3K, Src and Shc as outlined in figure 2.4 (43).

In addition, PTx sensitive $G\alpha_i$ subunits have been shown to activate ERK1/2 in a manner independent on $G\beta\gamma$, Ras and PI3K (44), but additional mechanistic details of this pathway are diffuse.

2.3.2.3 Activation of ERK1/2 through G_s-coupled receptors

Elevated cAMP levels can increase, decrease, or not influence the activation state of ERK1/2 in a cell type dependent manner. PKA phosphorylates and thereby inhibits Raf-1 (45), which is thought to be the main inhibitory mechanism of PKA on ERK1/2 activation.

In a number of cases, Rap1 has been implicated in the activation of ERK1/2 (21). Rap1 is activated by PKA or the cAMP-dependent GEF Epac which is specific for Rap1. Activated Rap1 is thought to induce activation of ERK1/2 through B-Raf and MEK (21).

In some cell types, however, B-Raf is not expressed at detectable levels, or is expressed but seemingly does not play a significant role in ERK1/2 activation. It has been proposed that the cytosolic kinases of the Src family are involved instead (46). Src may be activated via PKA phosphorylation or by direct interaction with β-arresin. This last case may indicate that activation of ERK1/2 could be linked to GPCR desensitization (47). In either case, activated Src can stimulate the recruitment of mSOS to RTKs or Pyk2 as described earlier, with subsequent activation of ERK1/2.

For the β_2-adrenoreceptor (β_2-AR) it has been published that PKA dependent phosphorylation of the receptor may cause it to switch coupling from G_s to G_i (24). This is thought to mediate not only desensitization, but also activation of ERK1/2 through a cascade involving β-arrestin, Src and dynamin. β-arrestin-bound β_2-AR is believed to form a complex with the EGF receptor, internalize with it and transactivate it (48). The hypothesis of G protein switching is, however, still a matter of controversy (25).

The G_s-coupled serotonin receptors 5-$HT_{4(b)}$ and 5-$HT_{7(a)}$ have been shown to activate ERK1/2 in a manner dependent on Ras and partly dependent on PKA, but independent of Rap1, but the link between PKA and Ras is yet to be elucidated (49).

2.4 Serotonin and its Receptors

Serotonin (fig. 2.5) was first discovered in the 1930's when Vittorio Erspamer was searching for substances capable of causing smooth muscle contraction. He named this substance enteramine, and it was later coined serotonin when in the late 1940's Irving Page isolated this vasoconstricting substance from serum. Its structure was reported in 1949, and in 1953, Betty Twarog discovered that serotonin was also operating as a neurotransmitter in the human brain.

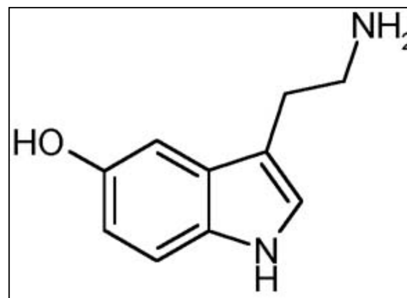


Figure 2.5 The chemical structure of serotonin

2.4.1 Serotonin (5-hydroxytryptamine, 5-HT)

Serotonin is a vasoactive biogenic amine which functions as hormone and a neurotransmitter with many effects throughout the human body. Serotonin is made from its precursor, the amino acid L-tryptophan by hydroxylation of the indole ring at C-5 and amino acid decarboxylation. Serotonin is either stored in vesicles or rapidly degraded by monoamine oxidase (MAO) to 5-hydroxyindoleaceticacid (5-HIAA).

About 90% of all serotonin in the body is found in enterochromaffine cells in the gastrointestinal mucosa, mainly in the duodenum. These cells produce and store serotonin

themselves, and are the main source of circulating serotonin. Basal enteric release of serotonin is augmented by mechanical stress such as stretching of the intestines by the passage of food. Such release of serotonin causes contraction of gastrointestinal (GI) smooth muscle cells, increases tone and facilitates peristalsis. The effect is mediated directly by serotonin receptors on smooth muscle cells and also indirectly through the enteric nervous system by stimulating ganglion cells.

Another important site of serotonin function is the central nervous system where it acts as a neurotransmitter. It is synthesized and stored by serotonergic nerons, of which there are about 300.000 in the human brain. Most of these originate from the raphe nuclei, but the extensive axonal projection system arising from these neurons bears a tremendous number of collateral branches so that the 5-HT system densely innervates nearly all regions of the CNS. Serotonin modulates and controls a wide range of behavioral and physiological processes including cognition, learning/memory, ingestion, blood pressure, perception of pain, nausea/vomiting, thermoregulation, circadian rhythms, aggression, depression and anxiety. Peripheral seretonergic neurons are found in many tissues and organs including the heart, kidneys, lungs, spleen urinary bladder and blood vessels in addition to the gastrointestinal tract.

In the cardiovascular system blood platelets are the major source of circulating serotonin. Blood platelets have no ability to synthesize serotonin themselves, but they use active transport mechanisms similar to that of serotonergic nerve endings to gather serotonin released from the enteric nervous system. Blood platelets can store this serotonin in granules and release it upon activation. Such activation is mediated by platelet contact and adhesion to subendothelial collagen via glycoprotein receptors interacting with fibrinogen, vonWillebrand factor, fibronectin and vitronectin. This leads to the classical thrombus formation and the release of many chemical mediators, among them serotonin. The effects of serotonin depend on the localization from which it is released. In the vascular system, serotonin can cause both vasodilation (mainly in arterioles and large veins), and vasoconstriction (mainly in large arteries and precapillary vessels), but many exceptions exist depending on exact vessel site (50). In the heart, serotonin mediates both cardioexcitation (increased inotropy and chronotropy) through a direct action (51), and cardiodepression indirectly via stimulation of the central nervous system, probably mediated through excitation of sensory afferent vagal nerves (51).

2.4.2 Classification of serotonin receptors

There are at least 14 subtypes of serotonin receptors. All of them are GPCRs, except the 5-HT_3 receptor which is an ion channel (52). Based on structural, functional and pharmacological criteria, the serotonin receptors are grouped into seven families (table 2.6).

Table 2.6 Overview of the human 5-HT receptor families

Family	Members	Isoforms*	Coupling	Intracellular effect
5-HT_1	1A, 1B, 1D, 1E, 1F	Not known	$G\alpha_{i/o}$	Inhibition of AC
5-HT_2	2A, 2B, 2C	Yes	$G\alpha_{q/11}$	Activation of PLC
5-HT_3	3A+3B (heteromeric), 3C	Yes	$Na^+/K^+/Ca^{2+}$	Depolarization
5-HT_4		4(a) - 4(h)	$G\alpha_s$	Stimulation of AC
5-HT_5	5A, (5B)	Not known	Unknown	Inhibition of AC?
5-HT_6		Not known	$G\alpha_s$	Stimulation of AC
5-HT_7		7(a) 7(b) 7(d)	$G\alpha_s$	Stimulation of AC

* produced by alternative splicing or mRNA editing.

Whereas most of the 5-HT receptors are encoded by a single exon and do not contain splice variants, the 5-HT_4 and 5-HT_7 receptors do (53). In addition, the 5-HT_{2C} receptor is a target for mRNA editing, producing several functional isoforms (54).

To discuss the enormous variety in function, localization and physiological effects of the serotonin receptors is beyond the scope of this thesis. In summary, the 5-HT_1 receptors are found mainly in the CNS, the 5-HT_2 receptors are found mainly in the CNS, skeletal/smooth muscle and kidneys, the 5-HT_4 receptors are found in the CNS, GI tract, vascular smooth muscle, urinary bladder and heart, and the 5-HT_6 receptor is found in the brain only. A more comprehensive outline of the 5-HT_7 receptors is the subject of chapter 2.4.4.

2.4.4 The human 5-HT_7 receptors

There are three human 5-HT_7 receptor splice variants, 5-$HT_{7(a)}$, 5-$HT_{7(b)}$ and 5-$HT_{7(d)}$. Structurally they differ only in their intracellular C-termini (55,53) (fig. 2.6). The receptors share only low sequence homology with the other members of the 5-HT receptor family (39% with the G_s-coupled 5-HT_6 receptor and 46% with the 5-HT_4 receptor), but functionally many similarities exist between the various G_s-coupled serotonin receptors.

The 5-$HT_{7(b)}$ (432 amino acids) is a truncated version of the 5-$HT_{7(a)}$ receptor (445 aa), whereas the 5-$HT_{7(d)}$ receptor (479 aa) is an extended variant with two extra putative phosphorylation sites, a PKC and a casein kinase II site respectively. A non-classical group II PDZ binding motif is present on the C-terminus of the 5-$HT_{7(b)}$ receptor, but the cellular significance of this is unknown (56). The phosphorylation sites and the PDZ motif could have important functional consequences, such as different G-protein-coupling efficiency or different susceptibility to desensitization.

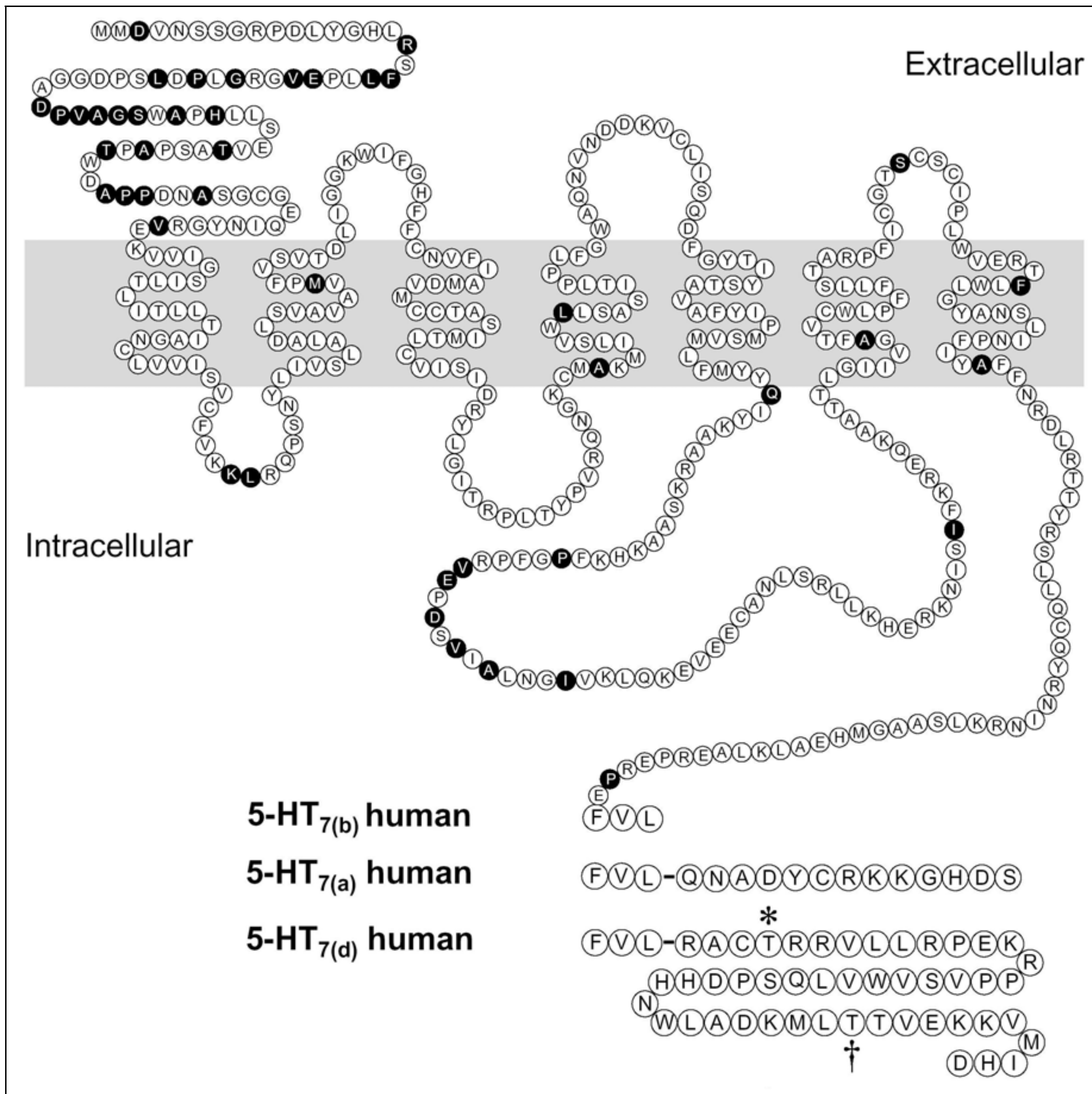


Figure 2.6 The human 5-HT_7 receptor splice variants. * putative PKC phosphorylation site, † putative casein kinase II phosphorylation site (adopted from Vanhoenacker et al. (60))

All the 5-HT_7 receptors modulate AC positively through $G\alpha_s$ with no detected difference between the splice variants (57,58). In fact, the various isoforms have been shown to share indistinguishable pharmacological and signal transduction properties, differing only somewhat in tissue distribution. Usually however, they are even co-localized in the same tissue, though at various relative abundance (58,59).

The 5-HT_7 receptors are believed to play an important regulatory role in CNS, especially in the limbic and cortical systems, as well as in the hippocampus and hypothalamus. Circadian rhythm and depression have been linked to 5-HT_7 receptor localization and function (60). In peripheral tissues, the receptors generally mediate smooth muscle

relaxation, and have been found in blood vessels, including the coronary arteries, the descending colon and the ileum.

In membranes from HEK293 cells stably or transiently expressing either of the three human 5-HT_7 receptor splice variants, basal AC activity is elevated higher than in non-transfected HEK293 cells (61), indicating some form of constitutive activity of the receptors.

2.4.4.1 Activation of ERK1/2 through 5-HT_7 receptors

Agonist stimulation of endogenously expressed 5-HT_7 receptors in cultured rat hippocampal neurons induces activation of ERK1/2 (62). This activation is not sensitive to PTx, and is therefore unlikely to involve $G_{i/o}$-proteins.

Activation of ERK1/2 has also been shown in COS7 and HEK293 cells transiently transfected with 5-$HT_{7(a)}$ receptors. This stimulation was dependent on Ras and Raf-1, but did not require transactivation of EGF receptors (49).

The complete mechanism by which ERK1/2 is activated by the 5-HT_7 receptors is yet to be elucidated (see also chapter 2.4.4.2)

2.4.4.2 The 5-HT_7 receptors and calcium

There are many kinds of Ca^{2+} channels in the body, but the variety of functions is beyond the scope of this thesis. A brief summary is presented in table 2.7.

Table 2.7 Summary of some calcium channels and transporters

Type	Main localisation	Main functions
RyR1 (calcium-induced)	Skeletal muscle, cerebellum	Ca^{2+} release from ER/SR
RyR2 (calcium-induced)	Cardiac muscle, brain	Ca^{2+} release from ER/SR
RyR3 (calcium-induced)	Brain, smooth muscle	Ca^{2+} release from ER/SR
IP_3R	Most nucleated cells	Ca^{2+} release from ER/SR
T-Type (low-voltage-activated)	Cardiac SA node, neurons, endocrine cells, smooth muscle.	Pacemaking, repetitive firing
L-Type (high-voltage-activated)	Smooth muscle, skeletal muscle, cardiac myocytes, endocrine cells, neurons.	Excitation-contraction coupling, hormone release, neurotransmitter release
Na^+/Ca^{2+} exchanger	Most nucleated cells, SR/ER, plasma membrane	Removal of cytoplasmic Ca^{2+}
Ca^{2+} ATPase	Most nucleated cells, SR/ER, plasma membrane	Removal of cytoplasmic Ca^{2+}

Several mechanisms may be responsible for increase in free intracellular calcium (Ca^{2+}_i) in HEK293 cells. Release of calcium from the endoplasmatic reticulum through the classical ryanodine receptors (RyR) is a possibility (63). The main function of RyRs is to amplify Ca^{2+}_i signals through calcium-induced calcium release, but may also be stimulated by protein phosphorylation mediated by PKA or calmodulin-dependent kinase II (64).

The 5-$HT_{7(a)}$ receptor has been shown to activate the neuron specific isoforms of adenylyl cyclase, AC1 and AC8 (65). These types of adenylyl cyclase are found in areas of the brain

(hippocampus and hypothalamus) where the 5-HT_7 receptors have been localized. It has been demonstrated previously that activation of the 5-$HT_{7(a)}$ receptor results in increases in Ca^{2+}_i, which is consistent with an increase in cAMP as a result of activation of the Ca^{2+}/CaM sensitive AC1 and AC8 (65). This effect however, was shown to be independent of PKC, phosphoinositide turnover and G_i-proteins (65). It could be that direct interaction with a Ca^{2+} channel occurs, possibly mediated by a PDZ domain as suggested for the β_2-AR (66).

In rat glomerulosa cells, activation of endogenous 5-HT_7 receptors is associated with an increase in AC activity and Ca^{2+} influx through T-type calcium channels (67). The link between the G_s coupled 5-HT_7 receptors and Ca^{2+} is very interesting in the perspective of ERK1/2 activation. It has been shown that Ca^{2+} plays an important role in stimulation of ERK1/2 through calmodulin, Src, Pyk2, PKC, Ras-GRF1 and CalDAG-GEFs. It can thus be envisioned that activation of the G_s-coupled 5-HT_7 receptors leads to an increase in both cAMP and Ca^{2+} with activation of ERK1/2 occurring as a consequence. Exactly how this is brought about is currently unknown and needs to be clarified.

2.5 Background and Purpose of the Present Study

With basis in the theories presented in the preceding chapters, the main aim of this thesis is to elucidate the signal transduction sequences leading from agonist binding to the human 5-HT_7 receptors to activation of ERK1/2. There are two incentives for doing this. First to better understand the function of these serotonergic receptor systems at a molecular level, as these receptors may play important roles in some pathological conditions. Secondly, and most importantly, to discern a possible model system for how G_s-coupled receptors in general mediate activation of mitogen activating protein kinases.

3 Methods

3.1 Amplification of DNA plasmids

When eucaryotic DNA plasmids are transformed into bacteria, they are replicated independently of procaryotic chromosomal DNA. Transformation can thereby be used to produce high numbers of plasmid construct copies, which in turn can be used in transfection procedures of human eucaryotic cells (*i.e.* HEK293)

3.1.1 pcDNA3.1 vector

We have used the pcDNA3.1(+) (V1) from Invitrogen extensively and is presented as an example of a DNA vector. Other vectors exibit similar qualities.

pcDNA3.1(+) is a 5428 bp vector derived from pcDNA3 and designed for high-level stable and transient expression in mammalian hosts. The human cytomegalovirus immediate-early (CMV) promoter provides high-level expression in most eucaryotic cells, including HEK293. In addition, the vector can replicate episomally in cell lines that are latently infected with SV40.

3.1.2 Plasmid propagation

The kit MaxiPrep from QIAGEN was used for amplification of different constructs.

- pcDNA3.1 with 5-$HT_{7(a)}$ receptor insert
- pcDNA3.1 with 5-$HT_{7(b)}$ receptor insert
- pcDNA3.1 with 5-$HT_{7(d)}$ receptor insert
- pMT2HA with Rap1GAP1 insert
- pCMV with RasN17 insert
- pCMV with RasV12 insert
- pKH3 with HA-Ras-GRF1-wt insert
- pEGFP-C2 encoding green fluorescence protein (GFP)
- pcDNA3.1(-) empty vector (V2)

The bacterial cells used for transformation are strains of *Escherichia coli*. These bacteria are pretreated with various chemicals to become competent for DNA transformation. In this thesis we used “TOP-10 Chemically Competent *E.coli*” from Invitrogen, and the transformation protocol used was called “TOPO One-Shot”.

3.1.3 TOPO One-Shot transformation

Competent cells are highly sensitive to changes in temperature or mechanical lysis caused by pipetting. Transformation should be started immediately following the thawing of the cells on ice. Mix by swirling or tapping the tube gently, not by pipetting.

Protocol:

1. Thaw, on ice, one 50 µl vial of One Shot cells for each ligation/transformation.
2. Add 1 to 10 µg plasmid DNA to 25 µl competent cells and mix gently.
3. Incubate the cells on ice for 30 min.
4. Heat shock the cells for exactly 30 s at 42 ºC without shaking.
5. Transfer the vial immediately to ice and incubate for 2 min without shaking.
6. Add 250 µl of SOC medium (room temperature).
7. Cap the tube tightly, shake horizontally at 37 ºC for 1 hour at 225 rpm.
8. Spread 50 – 100 µl of the transformation mixture on to an LB (Luria Bertani)-agarose-ampicillin (75 µg ampicillin/ml) plate.
9. Invert the plate and incubate overnight at 37 ºC.

3.1.4 QIAGEN maxiprep plasmid purification

The QIAGEN maxiprep plasmid purification kit is used for large-scale plasmid preparations. The cells are lysed using a NaOH and SDS containing solution (buffer P2) in the presence of RNase A (buffer P1). SDS solubilizes the phospholipid and protein components of the cell membrane, leading to lysis and release of cell contents. NaOH denatures chromosomal and plasmid DNA, as well as proteins. RNase A is used to digest liberated RNA as to minimize RNA contamination. An optimized lysis time will ensure that the plasmid DNA is released while the chromosomal DNA remains attached to the cell-wall. The lysates are neutralized by the addition of acidic potassium acetate (buffer P3). The high salt concentration causes potassium dodecyl sulfate (KDS) to precipitate, and the denatured proteins, chromosomal cell-wall-bound DNA and cellular debris become entrapped in salt-detergent complexes. Plasmid DNA on the other hand, being smaller and circular, renatures and remains in solution. Centrifugation removes the unwanted debris and the lysates are run through an anion-exchange column consisting of silica beads (100 µm particle size) with high density of diethylaminoethyl (DEAE) groups. DEAE is positively charged in an acidic environment and will interact with charged molecules, such as the phosphate groups on DNA. Degraded RNA, cellular proteins and other debris are not retained and appear in the flow-through fraction. Washing with a medium-salt buffer (buffer QC) will remove contaminants such as traces of RNA and proteins. Plasmid DNA is eluted using a high-salt buffer (buffer QF). The eluted plasmid DNA is desalted and concentrated by isopropanol precipitation.

All the buffers mentioned are part of the QIAGEN plasmid kit (see chapter 7.2.1.1)

Protocol:

1. Pick one colony from a LB-agarose plate described in 3.1.3 and resuspend it in 5 ml LB medium with 75 µg/ml ampicillin. Incubate the culture for 8 h at 37 ºC in a shaking incubator (300 rpm).
2. Transfer the starter culture into 500 ml LB medium with 75 µg/ml ampicillin. Grow at 37 ºC over night in a shaking incubator (300 rpm)
3. Transfer the overnight culture to a centrifuge tube and harvest the bacterial cells by centrifugation at 6000 x g (6000 rpm in a Beckman JA-10 rotor) for 15 min at 4 ºC. Pour the supernatant off until all medium has been drained.
4. Resuspend the pellet in 10 ml buffer P1 (containing RNase A) by vortexing and pipetting.
5. Add 10 ml of buffer P2, mix gently but thoroughly by inverting the tube 5-6 times and incubate at room temperature for exactly 5 min. (Do not vortex as this can result in shearing of the genomic DNA).
6. Add 10 ml of prechilled buffer P3, mix immediately but gently by inverting 4-6 times, and incubate on ice for 20 min. A fluffy white material forms and the lysate becomes less viscous.
7. Centrifuge at 20000 x g (12000 rpm in a Beckman JA-17 rotor) for 30 min at 4 ºC. Remove the supernatant containing the plasmid DNA promptly.
8. Recentrifuge the supernatant at 20000 x g (12000 rpm in a Beckman JA-17 rotor) for 15 min at 4 ºC. Remove the supernatant containing the plasmid DNA promptly.
9. Equilibrate a QIAGEN-tip 500 by applying 10 ml buffer QBT and allow the column to empty by gravity flow.
10. Apply the supernatant from step 8 to the QIAGEN-tip and allow it to enter the resin by gravity flow.
11. Wash the QIAGEN-tip with 2 x 30 ml buffer QC.
12. Elute DNA with 15 ml buffer QF into a centrifuge tube resistant to alcohol.
13. Precipitate DNA by adding 10.5 ml room-temperature isopropanol to the tube. Mix and centrifuge immediately at 15000 x g (10500 rpm in a Beckman JA-17 rotor) for 30 min at 4 ºC. Carefully decant the supernatant. Mark the tubes prior to centrifugation so as to ease the detection of the glassy DNA pellet.
14. Wash the DNA pellet with 5 ml of room-temperature 70% ethanol, and centrifuge at 15000 x g for 10 min. Carefully decant the supernatant without disturbing the pellet.
15. Air-dry the pellet and redissolve the DNA in 500 µl TE buffer.

3.1.5 Analysis of DNA Plasmids

3.1.5.1 Quantification of DNA by UV analysis

Spectrophotometrical quantification of plasmid DNA is performed by measuring optical absorption at λ = 230 nm, 260 nm and 280 nm (OD_{230}, OD_{260} and OD_{280}).

Asp, Glu, Asn, Gln, Arg, and His sidechains have absorption in the region 190 - 230 nm, whereas aromatic amino acids (Phe, Tyr and Trp) absorb UV-light around 280 nm. The Tryptophane band at 280 nm is clear-cut and specific, and this wavelength is therefore used generally to determine protein concentrations. Urea and phenol have absorption maxima at 230 nm.

Nucleotide spectra are complicated to analyze quantitatively because there are many non-bonded electrons with indistinct transitions giving a multitude of absorptions between 200 nm and 300 nm. All nucleotides do however have a λ_{max} near 260 nm which is fairly specific for the purine and pyrimidine bases. This wavelength can thus be used to estimate the nucleic acid concentration in a sample, and combined with the absorption at 230 nm and 280 nm an approximate purity can be calculated. Contamination by RNA on the other hand can not be detected by UV analysis as both RNA and DNA have absorption maxima at about 260 nm.

Pure DNA:

- OD_{260}/OD_{280} ratio ~ 1.8 - 1.9
- OD_{260}/OD_{230} ratio ~ 1.8 - 2.2

DNA absorbs light at 230 and 280 nm also, but to a lesser extent. Absorbance at 230 nm indicates contamination by urea, phenol or proteins, whereas absorbance at 280 nm denotes mainly protein contamination. A lower value of either ratio (*i.e.* a relatively lower absorbance at 260 nm) indicates contamination. Using OD_{260} to estimate concentration is only valid if the ratios are within their limits, otherwise the solution is not pure nucleic acid and the OD_{260} may include absorbance by other molecules.

Given these premises the total amount of DNA can be calculated using the formula:

$$\textbf{[DNA] (µg/µl) = } \mathbf{OD_{260}} \textbf{ x dilution / 20}$$

Protocol:

1. Tune the spectrophotometer to measure ODs at 230 nm, 260 nm and 280 nm.
2. Clean the quartz cuvettes with 6 M HCl and then dH_2O.
3. Dilute the plasmid sample 1:100 with a suitable solvent (*e.g.* TE pH 8.0 or dH_20). 1 ml total volume is sufficient for the cuvette.
4. Calibrate the apparatus using a blank containing only the solvent and then analyze the plasmid at the specified wavelengths. The readout should look like figure 3.1.

Sample	230 nm	260 nm	280 nm	260/230	260/280	Conc.
Ref	0.000	0.000	0.000			
1	0.126	0.270	0.152	2.14	1.78	1.352
2	0.211	0.426	0.238	2.03	1.79	2.132
3	0.225	0.480	0.262	2.14	1.83	2.401
4	0.115	0.250	0.135	2.18	1.85	1.250

Figure 3.1. Example of a spectrophotometer readout. The image shows an example of a readout from the Ultrospec 2100 Pro instrument providing data for 230, 260 and 280 nm. Column 3 shows the absorption at 260 nm, and from this we can derive the concentration in column 7. Columns 5 and 6 indicate the purity of the sample which in this case is acceptable.

3.1.5.2 Agarose gel analysis

Agarose gel electrophoresis can be used to distinguish and separate DNA fragments of different sizes. Gels with 1.0-1.3% agarose are used for separating small fragments (0.1-2.0 kilobases), whilst 0.6-1.0% agarose gels are used for separating larger fragments (2.0-10.0 kb). Due to the negatively charged phosphate groups, DNA molecules will be attracted to the cathode in an electrical field. DNA fragments of smaller sizes will migrate faster than those of larger sizes because they more readily travel through the polymer meshwork. This can thus be used in separating a mixture of fragments according to size. Also, supercoiled fragments will migrate faster than linearized fragments and open circles. To determine the size of the separated DNA molecules, they are compared to DNA marker fragments of known size run on the same gel. The gel is preloaded with ethidium bromide, a substance which intercalates between the basepairs of the DNA double helix, and the fragments are visualized using UV-light.

Protocol:

0.9% (w/v) agarose gel suitable for 0.5 – 7 Kb DNA.

1. Dissolve 1.80 g agarose in 200 ml TAE buffer by heating to the boiling point in a microwave oven.
2. Let the solution cool to 50-60 °C and add 20 µl of 2.5 mg/ml ethidium bromide. Pour the warm solution carefully into a gel mould fitted with the appropriate comb and remove air bubbles by the tip of a pipette. Allow the gel to set at room temperature.
3. Remove the comb, transfer the gel to an electrophoresis chamber and cover it completely with 1x TAE buffer.
4. Prepare the DNA samples with DNA loading buffer. Apply a DNA marker in the first lane and samples in the remaining wells.
5. Run the gel at 50-90 V.
6. Visualize the DNA in the gel under UV-light.

Samples from the QIAGEN maxiprep plasmid purification protocol described in section 3.1.4, can be analyzed using agarose gel electrophoresis. Samples from each step in the maxiprep protocol must be taken out for this purpose. The samples collected at the indicated steps may be analyzed on a 0.9 % (w/v) agarose gel to determine:

- Whether the growth and lysis conditions were optimal (Lysate supernatants (step 8, sample 1))
- Whether the efficiency of DNA binding to the QIAGEN resin was satisfactory (Flow-throughs (step 10, sample 2))
- Whether DNA was lost during the washing procedure (Wash-fractions (step 11, sample 3))
- Contents of DNA in the final eluate (Eluates (step 12, sample 4))

3.2 Culturing HEK293 Cells

HEK293 cells (fig. 3.2) are epithelial human embryonic kidney cells transformed with adenovirus 5 DNA (HAd5) established by F.L.Graham in 1977 (68). The cells are hypotriploid and form tumours in nude mice. The cells grow well both in serum-containing and serum-free medium, adhere to plastic surfaces and have a virtually infinite life span. HEK293 cells express several receptors that will mediate activation of ERK1/2 (*e.g.* the EGF receptor) However, serotonin receptors that mediate activation of ERK1/2 are not expressed. Therefore, HEK293 cells were chosen for studying activation of ERK1/2 mediated through specific serotonin receptors.

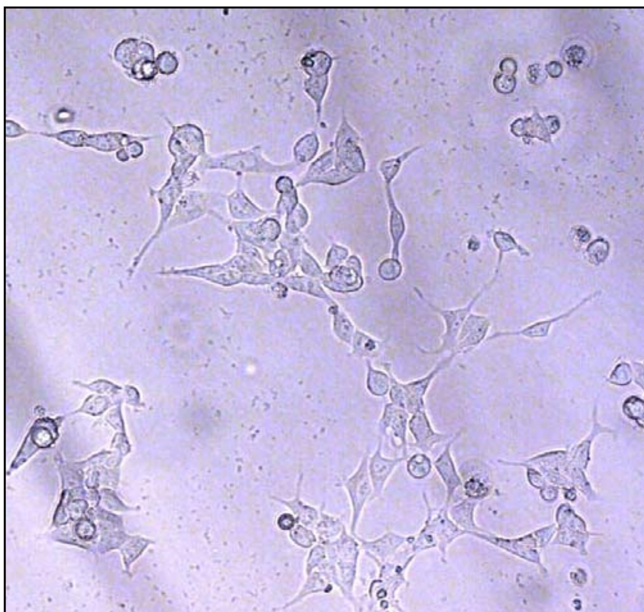


Figure 3.2 HEK293 cells growing in culture. Bright field filter, 20x objective (Olympus IX81).

3.2.1 Thawing cells

Protocol:

1. Remove one vial of cells (1 ml of cell suspension containing ~10^7 cells) from the liquid nitrogen tank. Thaw the suspension in a water bath at 37 ºC until only a tiny piece of ice is left.
2. In a LAF bench, using aseptic procedures, add the cell suspension to a 15 ml tube containing 5 ml prewarmed (37 °C) complete medium (DMEM with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin).
3. Centrifuge the suspension for 5 min at 160 g.
4. Resuspend the pellet in 10 ml prewarmed complete medium and transfer to a 100 mm cell culture plate.
5. Incubate cells at 37 ºC in a humified atmosphere of 5% CO_2 in air over night.
6. Split cells 1:4 and seed the cells on 100 mm cell culture plates.

3.2.2 Splitting cells

HEK293 cells grow poorly at low confluency (<20%) due to lack of cell contact and paracrine growth enhancers. At high densities (>90%) however, the cells tend to aggregate and form a very dense monolayer which is difficult to split. Confluent cells at 80-90% (one 100 mm dish containing approximately 10^7 cells) form a homogeneous monolayer and are readily transfected, thus 1:4 or 1:6 dilution is regarded as optimal when splitting these cells.

At 70-80% confluency the HEK293 cells are subjected to a treatment with trypsin-EDTA (0.5 mg/ml trypsin and 0.2 mg/ml EDTA in PBS). Trypsin is a proteolytic enzyme and combined with the calcium chelator EDTA, the cells are detached from the culture dish surface and can be seeded out on new plates.

Protocol:

1. In a LAF bench, using aseptic procedures, remove medium and wash once with 10 ml sterile 0.9% NaCl solution.
2. Add trypsin-EDTA to barely cover the cells (approximately 1.5 ml for a 100 mm culture dish), and incubate the dish at 37 ºC until the cells detach (1-2 min depending on cell confluency). A firm knock on the side of the plate once or twice will promote the detachment.
3. Add 4.5 ml preheated (37 ºC) complete medium to the plates to inhibit further effect of the trypsin-EDTA solution, and disperse the cells by pipetting. Transfer the cell solution to a 15 ml tube.
4. Centrifuge the cell suspension at 160 g for 5 min at room temperature.
5. Resuspend the cell pellet in 6 ml preheated (37 ºC) complete medium.
6. Seed the cells out on new plates at a ratio of 1:4 or 1:6 depending on the original confluency (about 2 million cells per plate).

3.2.3 Freezing cells

When ordinary cells freeze, their plasmamembranes disrupt and vital parts are damaged because of ice crystal formation. Cryoprotective Medium contains 15% DMSO, which penetrates the cells and prevents plasmamembrane rupture. A slow freezing process further augments survival of the cells. The ideal decrease in temperature for freezing cells is about 1 ºC per hour, but the process can't be too slow due to the toxicity of DMSO present in the cryo-medium.

Protocol:

1. When the cells are 60-70% confluent, remove the medium, wash the cells with 0.9% NaCl and treat the cells with trypsin-EDTA as previously described.
2. Suspend cells in 10 ml of complete medium.
3. Centrifuge the cells at 160 g for 5 min at room temperature.
4. Resuspend the cells in 0.5 ml complete medium (without antibiotics), put on ice and add 0.5 ml ice-cold Cryoprotective Medium containing 15% DMSO.
5. Transfer the cell suspension to a cryotube kept on ice.
6. Place the cryotube in a styrofoam box at –70 °C, to let the temperature slowly decrease over night. Transfer the frozen cells to liquid nitrogen for long term storage.

3.3 Cell Experiments

3.3.1 Transfecting cells

Transfection is a method used for introduction of exogenous DNA into eucaryotic cells. There are several techniques by which this can be accomplished, but for HEK293 cells the method employing Lipofectamine (Invitrogen) is widely used and works well, giving transfection rates of up to 60%. Lipofectamine is a 3:1 (w/w) liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE in water. The plasmid DNAs form complexes with the Lipofectamine reagent, these complexes are then able to cross the plasma membrane of the cells, and the plasmids can then be replicated and transcribed episomally.

Protocol (transient transfection)

1. On 35 mm tissue culture plates, seed $2x10^5$ cells in 2 ml DMEM with 10% FBS per plate, and incubate the cells at 37 ºC in a humidified atmosphere of 5% CO_2 in air, until the cells are 70-80% confluent (This will usually take 18- 24 h).
2. For each 35 mm dish of cells prepare the following solutions in sterile tubes:
   Solution A: Dilute 1 µg of DNA into 100 µl serum-free DMEM.
   Solution B: Dilute 9 µl of Lipofectamine reagent into 100 µl serum-free DMEM. The amount of Lipofectamine may need to be adjusted depending on cell line variations.

3. Pour solution B into solution A and mix gently. Incubate at room temperature for 30 min, then add 0.8 ml serum-free DMEM to the tube containing the DNA-lipid complexes. Mix gently.
4. Wash the cells once with 2 ml serum-free DMEM.
5. Overlay the transfection solution onto the washed cells.
6. Incubate the cells for 5 h at 37 ºC in a humidified atmosphere of 5% CO_2 in air.
7. Remove the transfection mixture and add 1.5 ml DMEM with 10% FBS per culture plate.
8. Replace medium 18-24 h after the start of the transfection.

3.3.1.1 Analysis of transfection rate

There are many factors affecting the rate of transfection, the most important being:

- Cell number and confluency of the cells (50-80%)
- Volume of Lipofectamine (2-20 µl)
- Amount of DNA (0.5-2 µg)
- Exposure time to transfection mixture (4-6 h)

The transfection rate can be determined empirically by using plasmids containing the gene encoding the green fluorescent protein (GFP), and the mentioned parameters may be adjusted to optimal values. GFP is a spontaneously fluorescent protein isolated from the jellyfish *Aequoria victoria*. It is comprised of 238 amino acids and forms a β-barrel with a fluorophore located on the central helix. Its wild-type absorbance/excitation peak is at 395 nm with a minor peak at 475 nm. The cells which have been subjected to successful transfection will consequently express GFP and the number of transfected cells can be visualized using fluorescent light and compared to the total number of cells (fig. 3.3)

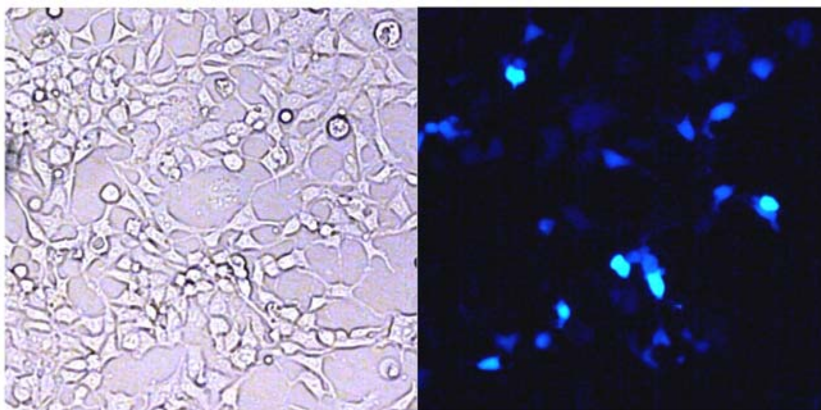


Figure 3.3 HEK293 cells transiently transfected with DNA plasmids encoding the green fluorescent protein. In the left panel: bright field 20x objective and right panel: fluorescence by GFP (wide blue filter) 20x objective (Olympus IX81).

3.3.2 Stimulating and harvesting

Transient or stable transfections with plasmids containing genes encoding either the 5-$HT_{7(b)}$ or the 5-$HT_{7(d)}$ receptor give us the opportunity to stimulate cells with serotonin, and thus observe what cellular signaling cascades are involved. A variety of other chemicals may be introduced to the cells to inhibit or induce signals, and combined with co-transfection with plasmids encoding constitutively active or dominant negative mutants of certain proteins in the signaling networks of inquiry, we can deduce what molecular mechanisms lay behind cellular effects.

Protocol:

1. Using the transiently transfected cells, replace the medium with serum-free DMEM 18 h prior to stimulation based on the experience that this results in the lowest possible basal ERK1/2 activation.
2. Carefully add the stimulation mixture (see table 3.1) into the medium and tilt the plate to make sure that the chemicals are evenly distributed in the medium.
3. Incubate for the desired period of time at 37 ºC in a humidified atmosphere of 5% CO_2 in air.
4. Remove the culture plates after stimulation and harvest the cells as described below.

Table 3.1 Concentrations of drugs and their vehicles

Drug	*Final conc.*	*Conc. of drug in vehicle (stock)*	*Vehicle*
5-HT	20 µM	10 mM	10 mM HCl
H89	20 µM	25 mM	DMSO
EGF	10 nM	5 µM	10 mM acetic acid
Thapsigargin	1 µM	1 mM	DMSO
BAPTA-AM	40 µM	50 mM	DMSO
Wortmannin	1 µM	1 mM	DMSO
CAI	20 µM	20 mM	DMSO
$GdCl_3$	20 µM	20 mM	dH_20
W-7	25 µM	30 mM	DMSO
KN-93	20 µM	25 mM	DMSO
PP2	20 µM	2 mM	DMSO
PP3	20 µM	2 mM	DMSO
8CPT-2Me-cAMP	30 µM	30 mM	10 mM Tris-HCl

The cells are harvested under denaturating and lysing conditions using the harvesting solution described under Materials. SDS is a strong detergent which disrupts the cell membranes and dissolves membrane proteins, ensuring also protein denaturation. Na_3VO_4 (sodium orthovanadate) is added as a potent general inhibitor of phosphatases, including phosphotyrosine phosphatases. Vanadate thus prevents dephosphorylation of phosphorylated proteins.

Protocol:

1. Rapidly remove the stimulatory medium
2. Place the dish immediately on ice and wash once with ice cold PBS.
3. Remove the PBS, place the dish upside down on paper towels to absorb excess fluids and add 200 µl cold harvesting solution onto the plate.
4. While keeping the plate on ice, scrape the cells from the bottom of the dish using a plastic cell scraper.
5. Transfer the cell lysate to a sterile 1.5 ml tube.
6. Shear the lysate six times through a 26 gauge needle.
7. Snap freeze the sheared lysate in liquid nitrogen.
8. Thaw the cell lysate at room temperature and let it settle on ice for 10 min.
9. Centrifuge the cell lysate at 11000 g (14400 rpm in the Eppendorf 5402 centrifuge, F-45-18-11 Rotor) for 7 min at 4 ºC.
10. Transfer the supernatant to a new sterile 1.5 ml tube and discard the pellet.
11. Snap freeze the sample in liquid nitrogen and keep at -70 ºC for long term storage. Alternatively, remove 3 x 5 µl of the sample to quantify the protein concentration using the BC assay protein quantification kit using BSA as standard. This to reduce the number of thawings and thus minimize the likelihood of dephosphorylation and degradation of proteins in the sample.

3.4 Analysis of Experiments

3.4.1 Protein quantification

To determine the amount of protein in each sample, BC (bicinchoninic acid) Assay: Protein Quantitation Kit from Uptima and a Perkin Elmer HTS 7000 Bio Assay Reader was used. Proteins reduce Cu^{2+} in alkaline solutions to Cu^{+} and two molecules of BCA interact with each molecule of Cu^{+} forming a water soluble red complex with an absorption maximum of 562 nm (fig. 3.4). Absorbance is proportional to the initial protein concentration between 1-20 µg/ml, allowing for spectrophotometric quantification of protein in aqueous solutions.

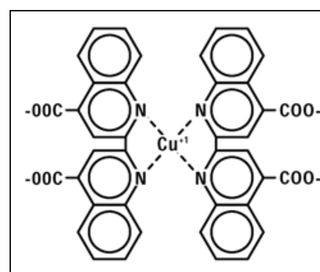


Figure 3.4 Bicinchoninic acid interacting with Cu^{+}

Protocol:

1. Load a flat bottom 96-well microtiterplate with BSA protein standard, control and samples according to figure 3.5.
2. Add 50 parts of BCA reagent A and one part of BCA reagent B to a tray, mix thoroughly and load 250 µl of this solution to each well in the microtiterplate.
3. Tape a sheet of adhesive plastic over the wells, and shake the plate gently for approximately 1 minute. Incubate for 30 min at 37 ºC.
4. Remove the adhesive plastic and let the plate settle to room temperature for a couple of min.
5. Analyse the samples using a plate spectrophotometer at a wavelength of 570 nm.
6. Ensure that the standard deviation of the standard curve is below 0.995. If not a new plate must be set up.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	s1	s1	s1							
B	125	125	s2	s2	s2							
C	250	250										
D	500	500										
E	750	750										
F	1000	1000										
G	1500	1500										
H	C	C										

Figure 3.5 Loading of microtiterplate for measurement of protein by the BCA method. The protein standards (µg/ml) are added as doublets with 20 µL in each well, an albumin control (C) can be added optionally as a doublet with 5 µL in each well and the cell lysates are added as triplets with 5 µL in each well.

3.4.2 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins of various sizes in a sample.

Sodium dodecyl sulphate (SDS) is a strong anionic detergent which wraps around the polypeptide backbone and disrupts the bonds that make up the three dimensional conformation of proteins. β-mercaptoethanol is also added to reduce any disulphide bridges within the structures. The samples are heat treated at 95ºC for 5 min to facilitate the denaturation process. In essence, the denaturated polypeptides become rods covered with negatively charged SDS. The negative charge is proportional to the length of the protein, and since the length is proportional to the mass for endogenous proteins, electrophoresis through a polyacrylamide gel can be utilized to separate them according to size. The intrinsic charge of the polypeptides is of marginal importance as the polypeptides are completely covered with the negative SDS. The proteins migrate toward the positive pole with a rate of migration dependent only on their size. The smallest fragments will travel furthest since they are least obstructed by the dense polyacrylamide network in the resolving gel.

3.4.2.1 Casting of polyacrylamide gels

The highly cross-linked gel of polyacrylamide serves as the inert matrix through which the denatured SDS-covered proteins migrate. The gel is prepared by polymerization from monomers, and the density (pore size) of the gel can be adjusted to suit the size of the proteins that are going to be separated. A 10% gel is optimal for separating p44 and p42 MAPK (the ERK1/ERK2 assay).

Protocol:

1. Wash the glass plates (one long and one short) and the spacers with water and 70% EtOH, repeat the procedure twice to ensure that the surfaces are clean.
2. Assemble the gel sandwich in the casting stand. Ensure that the clamp screws are tightened and that the plates are adjusted to avoid leakage.
3. Insert a comb and mark the glass plate about one centimeter below the comb's perimeter.
4. Mix the ingredients for the resolving gel of appropriate acrylamide concentration gently, ensuring no air bubbles form. Pour into the glass plate assembly carefully and overlay gel with 1-butanol to ensure a flat surface and to exclude air. Wash off 1-butanol with water after gel has set (1 hour).
5. Remove superfluous water with filter paper and insert the comb. Mix the ingredients for the stacking gel, and pour on top of the set resolving gel. Remove the comb after the stacking gel has polymerized (30 min), assemble the gel sandwich into the electrophoresis tank, and fill with Running Buffer.

3.4.2.2 Separation of proteins by gel electrophoresis

Protocol:

1. Transfer a predefined amount of protein (usually between 10 and 20 μg) to a sterile 1.5 ml tube.
2. Add the correct volume of diluent (Harvesting Solution) to ensure equal volume of each sample.
3. Add 1/4 of the sample volume of 4x Loading Buffer activated with 10% β-mercaptoethanol and vortex.
4. Heat the sample at 95 ºC for 5 min, centrifuge at 14400 rpm (11000 g) for 1 minute and vortex again. The finished protein loading samples can be frozen in nitrogen and stored at -20 ºC for later use, or can be loaded directly onto a polyacrylamide gel.
5. For each gel, prepare a protein marker by adding 7 μg Precision Plus Protein Standards, Dual Color to a 1.5 ml tube. Add 3 μl 4x Loading Buffer and 2 μl dH_20. Vortex the marker and it is ready to be loaded onto the polyacrylamide gel.
6. Load the protein marker and the samples onto the gel (12 μl per well)
7. Run the samples through the stacking gel at a constant current of about 15-20 milliamperes per mini-gel for approximately 30 min.
8. Run the samples through the resolving gel at a constant current of about 40-45 milliamperes per gel until optimal separation is achieved.

3.4.3 Western blotting

After electrophoresis of the proteins, the gel is subjected to electroblotting. The proteins negatively charged by SDS, can readily be transfered to a PVDF membrane in an electrical field.

Protocol:

1. Remove the gel from the glass plates and place it onto a blotting mesh folder containing a pad and three pieces of filterpaper (Whatman 3MM) (pretreated by soaking in 1x Blotting Buffer).
2. Place an activated (15 second pretreatment in methanol, then washed with 1x Blotting Buffer) PVDF membrane on the gel, ensuring that no air bubbles remain. Place three filterpapers on top of the membrane, as before.
3. Close the mesh folder and place it in the blotting cassette, and place this in the blotting container, already filled with 700 ml pre-chilled (4 ºC) 1x Blotting buffer. Make sure the membrane is orientated towards the cathode. Add a magnetic stir bar and a cartridge of ice to keep the system chilled through the blotting process.
4. Attach a power source, and perform the electroblotting at 400 mA for at least 45 min. The membrane can then be subjected to detection by immunofluorescence. If immunostaining is to be performed immediately, rinse the membrane in distilled water, if staining is to be performed at a later time, place membrane on a paper towel and allow the membrane to dry. The membrane can then be activated again by placing for 1 minute in pure methanol and then washed in dH_20 for 5 min.

3.4.4 Immunodetection

This is a highly specific method for detection of polypeptides on a Western blot membrane. A limitation to the method is that it requires specific antibodies, which can bind to the protein of interest. In these studies the proteins of main interest have been ERK1/2 (p44/p42) and their phosphorylated counterparts ppERK1/2 (see table 3.2). The total amount of ERK1/2 provides an internal control, and the amount of phosphorylated ERK1/2 in each sample must be correlated to the amount of non-phosphorylated ERK1/2 in the same sample before conclusions can be made.

The principle of immunodetection is the use of primary and secondary antibodies. The membranes are first subjected to a solution containing primary antibodies which bind specifically to the proteins of interest. A secondary antibody solution is then applied which binds to the Fc portion of the primary antibody. The secondary antibodies are conjugated with the enzyme horseradish peroxidase (HRP) which catalyzes the breakdown of luminol, thus emitting light. Our chemiluminescence reagent (Pierce SuperSignal West Dura Extended Duration Substrate) was composed of two components, one being luminol with enhancing reagent (a phenol derivative), the other being a peroxide solution, which were mixed 1:1 before use.

Protocol:

1. Incubate the membrane in 5% dried milk solution in PBS containing 0.05% Tween-20 at room temperature for 1 h, to non-specifically block the membrane.
2. Place the membrane in 8 ml of the dried milk solution containing the primary antibody at proper dilution. Incubate over night at 4 ºC with gentle tilting.
3. Remove the primary antibody solution and wash the membrane 3x10 min in PBS containing 0.05% Tween.
4. Incubate the membrane for 1 h at room temperature with gentle tilting in a solution containing the secondary antibody at proper dilution.
5. Remove the secondary antibody solution and wash the membrane 3x20 min in PBS containing 0.05% Tween.
6. Rinse the membrane in dH_20 to remove Tween-20.
7. Remove excess water by placing the membrane gently on a paper tissue.
8. Incubate the membrane for 5 min in 5 ml chemiluminescence reagent containing the HRP substrate (Pierce SuperSignal West Dura Extended Duration Substrate), 1:1 mixture as described above.
9. Place the membrane between two sheets of transparent paper, and expose for a suitable time (*see table 3.2*) in a darkroom (*e.g.* the UVP Epi Chemi II Darkroom).
10. An optical densitometric scanning may be performed of the digital image obtained from the developed membrane to get a quantitative result band intensities.

Table 3.2 Antibody concentrations for the ERK1/2 assay
(all antibodies were diluted in a solution of 5% dried milk in PBS with 0.05% Tween).

Phosphorylated ERK1/2	**Ab-dilution**	**Exposure time**
Primary antibody (Anti-ppERK1/2)	1:2000	
Secondary antibody (Anti-Mouse)	1:2000	4-7 min
Total ERK1/2	**Ab-dilution**	**Exposure time**
Primary antibody (Anti-ERK1/2)	1:10000	
Secondary antibody (Anti-Rabbit)	1:10000	1-3 min

Table 3.3 Antibody concentrations for the Ras-GRF1 assay
(all antibodies were diluted in a solution of 5% dried milk in PBS with 0.05% Tween).

pRas-GRF1	**Ab-dilution**	**Exposure time**
Primary antibody (Anti-pRas-GRF1)	1:1000	
Secondary antibody (Anti-Rabbit)	1:1000	4-7 min
Total Ras-GRF1	**Ab-dilution**	**Exposure time**
Primary antibody (Anti-Ras-GRF1)	1:2000	
Secondary antibody (Anti-Rabbit)	1:2000	4-7 min

3.4.4.1 Stripping of membranes

Membranes incubated with primary and secondary antibodies should normally be stripped before they are subjected to a new set of antibodies. When stripping the membrane, the antibody complex will detach together with a small amount of protein, and thus stripping can not be performed indefinitely.

Protocol:

1. Activate dry membranes in pure methanol for 15 s, wash in dH_20 for 5 min.
2. Incubate the membrane in 0.5 M NaOH for 5 min at room temperature.
3. Wash 2x5 min in dH_20.
4. Reprobe with a new antibody as described in section 3.4.4.

3.5 Direct cytoplasmic calcium measurements

Fura-2 is a fluorescent calcium chelator and can be used as a probe to measure intracellular calcium concentrations. It is provided as a cell permeable AM (acetoxymethyl) ester which is hydrolysed by esterases in the cytosol and trapped there. The spectral properties of fura-2 differ between its free form when Ca^{2+} concentrations are low (excitation max. 335 nm and emission max. 505 nm) and its calcium bound form when Ca^{2+} concentrations are high (excitation max. 362 nm and emission max. 512 nm). Since both fluorophors emitt fluorescent light with a wave length of about 510 nm after excitation, changes in calcium concentrations can be determined by imaging the cells at both 340 nm when calcium-bound fura-2 emitts light, and 380 nm when free fura-2 emitts light. The ratio 340/380 nm is used to reflect the proportion of Ca^{2+} bound to fura-2, and this correlates with the free cytoplasmic calcium concentration.

Protocol:

1. Mount the 1 cm^2 circular polyethylene wells with glass bottom and sterilize them with UV-light.
2. Coat the wells using 200 µl of 50 µg/ml acid soluble collagen type VII from rat tale (type 1). This is equivalent to 12.7 $\mu g/cm^2$. The wells are then dried in a LAF-bench over night and can be stored at 4 ºC.
3. Use transfected HEK293 cells from a 50-70% confluent 10 cm plate and collect the cells as described in chapter 3.2.2. Count the cells and dilute them with UltraCulture (with 10% PS and 10% Glutamine) so that about 75000-100000 cells are added to each well, a total of 500 µl cell suspension pr. 10 mm well.
4. Incubate the cells at 37 ºC in a humified atmosphere of 5% CO_2 in air until they are 50-75 % confluent.
5. Remove UltraCulture and wash the cells with 500 µl preheated HPPS buffer.
6. Expose the cells to 500 µl room temperature Fura-2/AM Loading Mixture (corresponding to 5 µM Fura-2) for 20 min at room temperature.
7. Wash the cells once with 400 µl HPPS buffer and add 400 µl HPPS buffer as the final volume before stimulation. Locate cells in the microscope and commence calcium measurements at once. 100 µl of the desired stimulation mixture is added after 30 s of recording the cells.

4 Results

The work on this thesis is based primarily on the original paper by Norum et al. (49) and is part of a ongoing project in Dr. Levy's group where the human G_s-coupled serotonin receptors, 5-$HT_{7(a)}$, 5-$HT_{7(b)}$, 5-$HT_{7(a)}$, 5-$HT_{4(a)}$ and 5-$HT_{4(b)}$ are investigated concurrently.

The majority of the results presented here were conducted with the 5-$HT_{7(b)}$ receptor, either in transiently or stably transfected HEK293 cells. The KB1 clone of the stably transfected HEK293 cells has been used. Unless otherwise stated in the figure legends, the cells have been harvested after stimulation, samples have been prepared of the cell lysates and these have been separated on SDS-PAGE and electroblotted over to PVDF-membranes before incubation with antibodies and subsequent immunoassay for detection of proteins, as described in detail under Methods.

4.1 Activation of ERK1/2

4.1.1 The 5-$HT_{7(b)}$ and 5-$HT_{7(d)}$ receptors activate ERK1/2

HEK293 cells do not endogenously express receptors that mediate activation of ERK1/2 subsequent to treatment with 10 μM 5-HT (data not shown). We have previously shown that the human serotonin receptors 5-$HT_{4(b)}$ and 5-$HT_{7(a)}$ mediate activation of ERK1/2 in HEK293 cells. We wanted to investigate whether the human serotonin receptors 5-$HT_{7(b)}$ and 5-$HT_{7(d)}$ mediate activation of ERK1/2 in a similar fashion. High resolution time courses showed that these receptors mediate phosphorylation of ERK1/2 (fig. 4.1, 4.2, 4.3 and 4.4) with maximal phosphorylation occuring after 4 - 7.5 min of 5-HT stimulation.

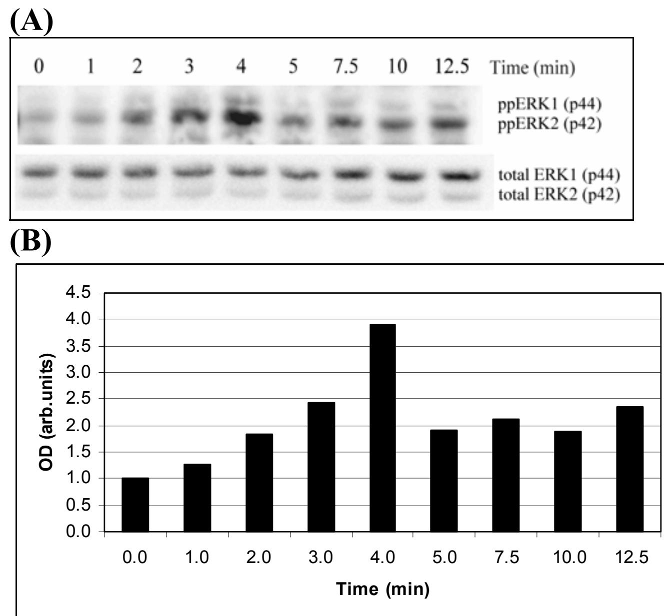
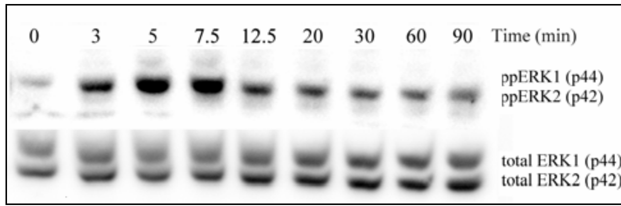


Figure 4.1 Activation of ERK1/2 through 5-$HT_{7(b)}$ receptors. A, HEK293 cells transiently transfected with plasmids encoding 5-$HT_{7(b)}$ receptors were treated with 5-HT (10 μM) for the indicated periods of time and assayed for detection of phosphorylated ERK1/2 as described in Methods. Proteins were separated on 10% SDS-PAGE and electroblotted over to PVDF-membranes before incubation with antibodies. Western blots were probed with phospho-specific ERK1/2 antibodies (*ppERK1/2*; *top panel*) and subsequently probed with anti-ERK1/2 antibodies (*ERK1/2*; *bottom panel*) to confirm equal loading. **B,** the same data is presented in a bar diagram based on optical densitometric scanning.

(A)



(B)

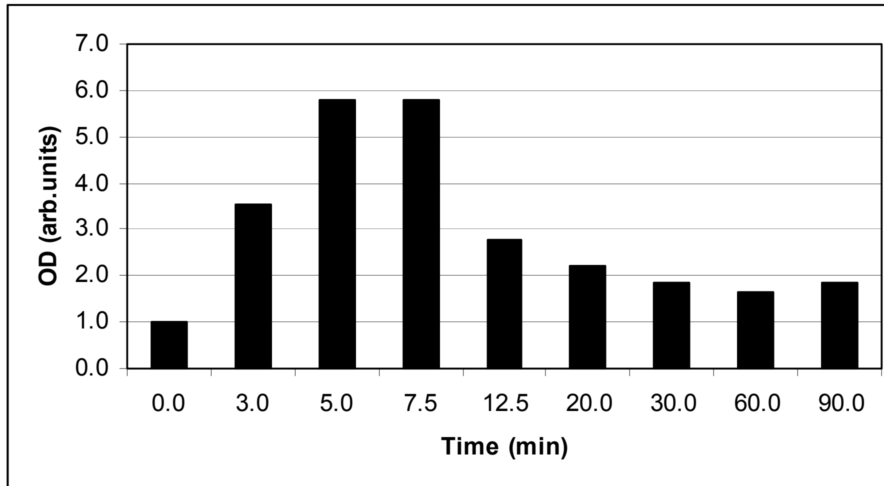


Figure 4.2 Activation of ERK1/2 through 5-$HT_{7(b)}$ receptors. HEK293 cells transiently transfected with plasmids encoding 5-$HT_{7(b)}$ receptors were treated with 5-HT (10 μM) for the indicated periods of time. The cells were harvested and processed, and the data presented as described in fig. 4.1.

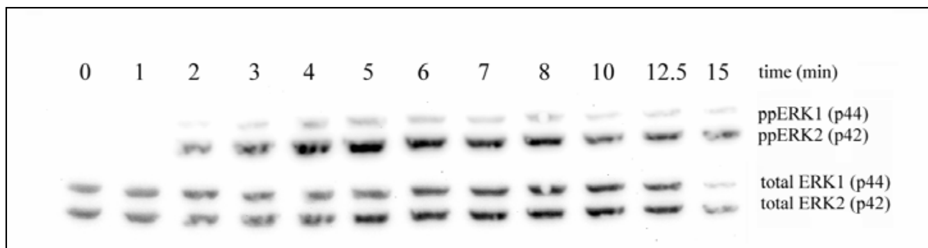


Figure 4.3 Activation of ERK1/2 in HEK293 cells stably expressing 5-$HT_{7(b)}$ receptors (KB1 cells). KB1 cells were treated with 5-HT (10 μM) for the indicated periods of time. The cells were harvested and the samples were processed as described in fig. 4.1.

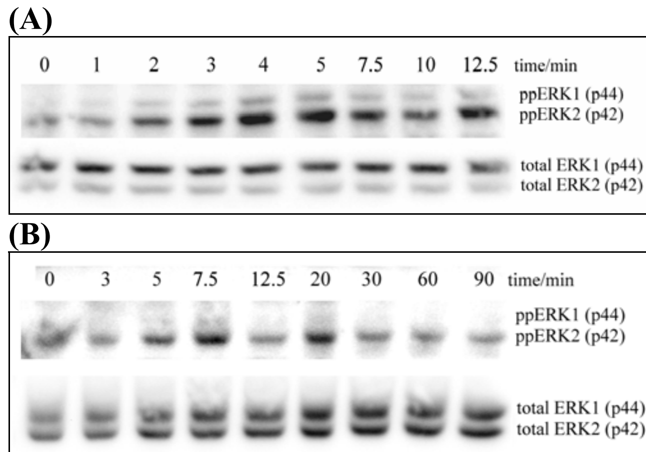


Figure 4.4 Activation of ERK1/2 through 5-HT$_{7(d)}$ receptors. A and **B,** HEK293 cells transiently transfected with plasmids encoding 5-HT$_{7(d)}$ receptors were treated with 5-HT (10 μM) for the indicated periods of time; **A**, high resolution and **B**, extended time courses. The cells were harvested and processed as described in fig. 4.1.

4.1.2 The role of Ras in ERK1/2 activation

Activation of ERK1/2 mediated through 5-HT$_{4(b)}$ and 5-HT$_{7(a)}$ receptors is dependent on Ras (49). Dominant negative and constitutively active constructs of Ras, RasN17 and RasV12 respectively, were used to elucidate the role of Ras in the activation of ERK1/2 through 5-HT$_{7(b)}$ and 5-HT$_{7(d)}$ receptors. HEK293 cells were cotransfected with 5-HT$_7$ receptors and RasN17 or RasV12 and the cells were treated with 5-HT prior to measurements of phosphorylated ERK1/2. The presence of RasN17 inhibited the 5-HT induced activation of ERK1/2 mediated through 5-HT$_{7(b)}$ (fig. 4.5A) and 5-HT$_{7(d)}$ (fig. 4.5B) receptors. Only weak or no additional phosphorylation of ERK1/2 was seen when cells cotransfected with 5-HT$_7$ receptors and RasV12 or RasN17 were treated with 5-HT.

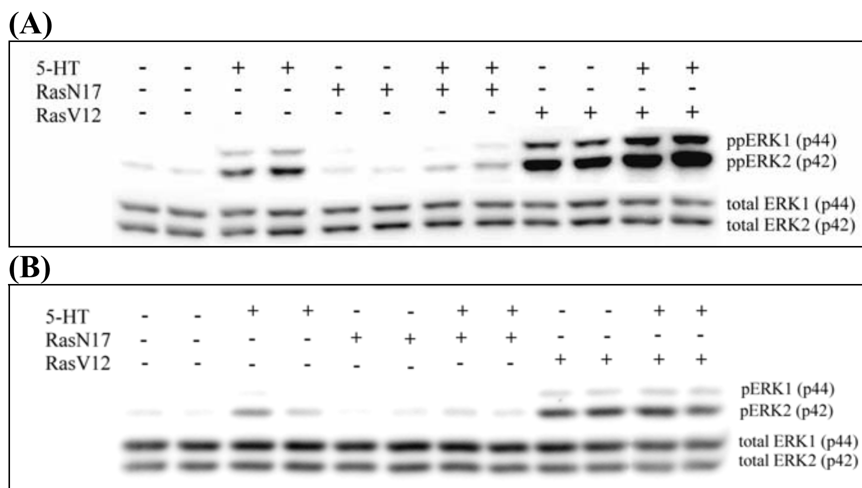


Fig. 4.5 Activation of ERK1/2 through 5-HT$_{7(b)}$ and 5-HT$_{7(d)}$ receptors is dependent on Ras. A, HEK293 cells were transiently cotransfected with plasmids encoding 5-HT$_{7(b)}$ or **B**, 5-HT$_{7(d)}$ receptors and empty pcDNA3.1(-) vector, dominant negative Ras (RasN17) or constitutively active Ras (RasV12) as indicated. The cells were treated with 5-HT (10 μM) or vehicle for 5 min, and harvested and processed as described in fig. 4.1. Panels **A** and **B** each show representative blots of two experiments.

4.1.3 The role of cAMP in ERK1/2 activation

The 5-HT_7 receptors couple to $G\alpha_s$ which results in elevated intracellular levels of cAMP. The plasma membrane permeable cAMP analogue 8-Br-cAMP is able to induce activation of ERK1/2 in many cell types, including HEK293 (69). It's thus liable to assume that the observed activation of ERK1/2 in our system is at least in part dependent on cAMP. HEK293 cells were cotransfected with plasmids encoding 5-$HT_{7(b)}$ receptors and hPDE4D2, a constitutively active human phosphodiesterase, and the phosphorylation of ERK1/2 was determined with Western blots. The 5-HT-induced phosphorylation of ERK1/2 was severely reduced in the presence of hPDE4D2 (fig. 4.6), suggesting that activation of ERK1/2 is dependent on cAMP.

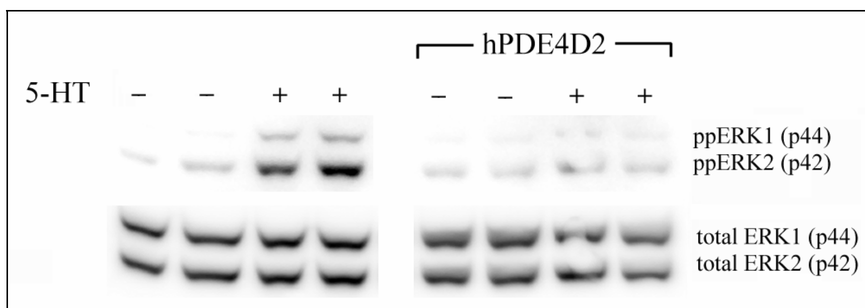


Fig. 4.6 Activation of ERK1/2 through 5-$HT_{7(b)}$ receptors is dependent on cAMP. HEK293 cells were transiently cotransfected with plasmids encoding 5-$HT_{7(b)}$ and the human PDE4D2. The cells were treated with 5-HT (10 µM) or vehicle for 5 min, and harvested and processed as described in fig. 4.1. The figure shows a representative blot of three independent experiments.

4.1.4 The role of PKA in ERK1/2 activation

Activation of ERK1/2 mediated through the 5-$HT_{4(b)}$ and 5-$HT_{7(a)}$ receptors was partly inhibited in the presence of H89, a PKA inhibitor (49). H89 and a construct encoding PKI, an inhibitory peptide for PKA, were employed to elucidate the role of PKA in the activation of ERK1/2 through 5-$HT_{7(b)}$ receptors. HEK293 cells transiently transfected with 5-$HT_{7(b)}$ receptors or stably transfected KB1 cells were subjected to preincubation with H89.

The effect of H89 is dubious: It did not interfere with ERK1/2 activation in five experiments of which three were conducted with the KB1 cells and two with transiently transfected cells (fig. 4.7A). It did however interfere with ERK1/2 activation to various degrees in four experiments of which two were conducted with the KB1 cells and two with transiently transfected cells (fig. 4.7B)

HEK293 cells were cotransfected with 5-$HT_{7(b)}$ receptors and PKI. In two consecutive experiments the presence of PKI did not inhibit activation of ERK1/2 (fig. 4.8A), but it interfered partially in one experiment (fig. 4.8B).

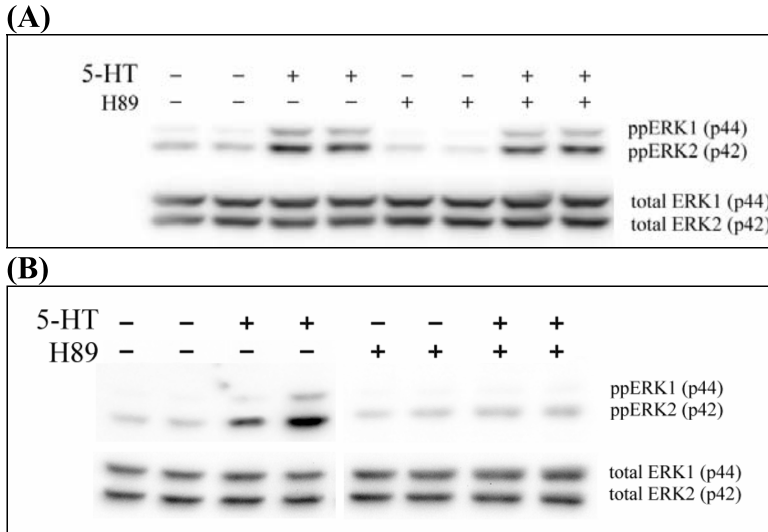


Figure 4.7 Variable effect of the PKA inhibitor H89. A, KB1 cells or **B**, HEK293 cells transiently transfected with $5\text{-HT}_{7(b)}$ receptors were pretreated with the PKA inhibitor H89 (20 μM) for 25 minutes prior to 5 min treatment with 5-HT (10 μM) or vehicle as indicated. The cells were harvested and processed as described in fig. 4.1. Representative blots of both results are shown in panels **A** and **B**.

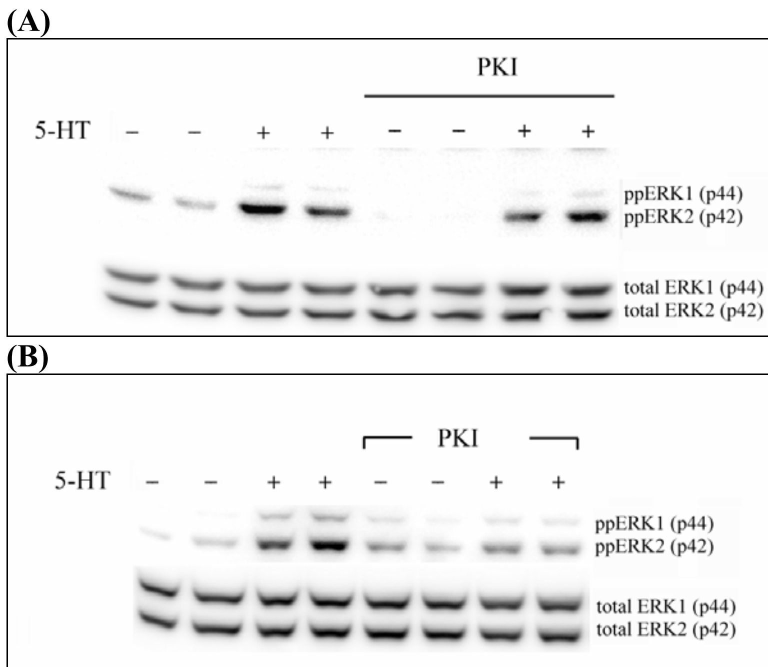


Figure 4.8 Variable effects PKI overexpression. A and **B,** HEK293 cells transiently cotransfected with plasmids encoding $5\text{-HT}_{7(b)}$ receptors and PKI were treated with 5-HT (10 μM) or vehicle for 5 minutes. The cells were harvested and processed as described in fig. 4.1. Representative blots of both results are shown in panels **A** and **B**.

4.1.5 The role of Epac and Rap1 in ERK1/2 activation

The Rap1 specific exchange factor Epac is activated by cAMP and has been proposed to have a role in the activation of ERK1/2 via certain G_s-coupled receptors. The proposed sequence is: cAMP → Epac/PKA → Rap1 → B-Raf → MEK → ERK.

Activation of ERK1/2 mediated through 5-$HT_{4(b)}$ and 5-$HT_{7(a)}$ receptors occurs independently of Rap1 (49). The Epac specific membrane permeable cAMP-analogue, 8-(4-chloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8CPT-2Me-cAMP) and a Rap1 specific GAP (Rap1GAP1) were used to determine the role of Epac and Rap1 in the activation of ERK1/2 through the 5-$HT_{7(b)}$ receptors.

The presence of 8CPT-2Me-cAMP did not activate ERK1/2 (fig. 4.9) and cotransfection with Rap1GAP1 did not interfere with ERK1/2 phosphorylation (fig 4.10), indicating that neither Epac nor Rap1 play any major roles in the activation of ERK1/2 through 5-$HT_{7(b)}$ receptors.

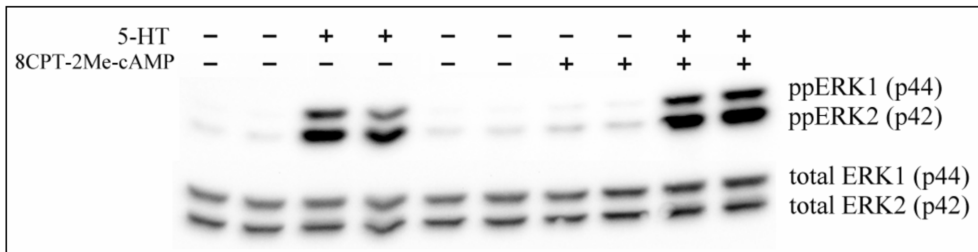


Figure 4.9 Activation of ERK1/2 through 5-$HT_{7(b)}$ receptors is independent of Epac. KB1 cells stably expressing 5-$HT_{7(b)}$ receptors were treated with 5-HT (10 µM) or 8CPT-2Me-cAMP (30 µM) for 5 min as indicated. The cells were harvested and processed as described in fig. 4.1. The figure shows a representative blot of three experiments.

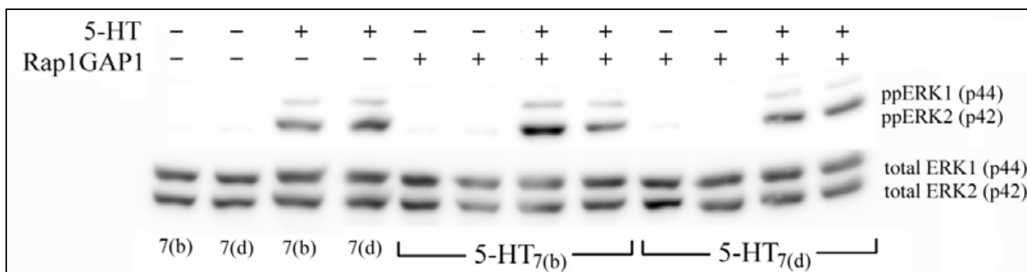


Figure 4.10 Activation of ERK1/2 through 5-$HT_{7(b)}$ and 5-$HT_{7(d)}$ receptors is independent of Rap1. HEK293 cells were transiently cotransfected with plasmids encoding 5-$HT_{7(b)}$ or 5-$HT_{7(d)}$ receptors, and Rap1GAP1 as indicated. The cells were treated with 5-HT (10 µM) or vehicle for 5 min. The cells were harvested and processed as described in fig. 4.1. The figure shows a representative blot of two experiments.

4.1.6 The role of PI3K in ERK1/2 activation

Several studies have implicated PI3K in a pathway mediating receptor induced activation of ERK1/2 (42,70). Wortmannin is a fungal metabolite that acts as a potent, selective, cell-permeable and irreversible inhibitor of PI3K. Preincubation with wortmannin did not interfere with the phosphorylation of ERK1/2 (fig. 4.11) indicating that ERK1/2 activation through 5-$HT_{7(b)}$ receptors occurs independently of PI3K.

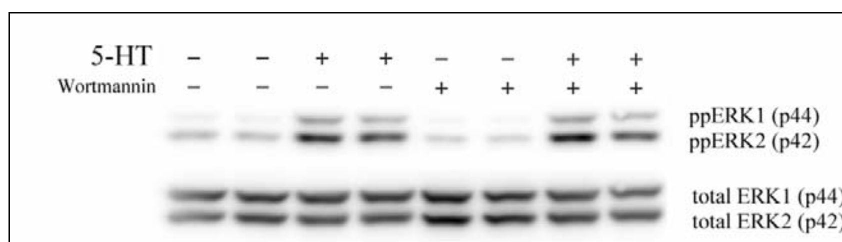


Figure 4.11 Activation of ERK1/2 is not blocked by a PI3K inhibitor. KB1 cells stably expressing 5-$HT_{7(b)}$ receptors were pretreated with wortmannin (1 µM) for 25 min prior to treatment with 5-HT (10 µM) or vehicle for 5 min. The cells were harvested and processed as described in fig. 4.1. The figure shows a representative blot of four experiments, two with KB1 cells and two with HEK293 cells transiently transfected with 5-$HT_{7(b)}$.

4.1.7 The role of Src in ERK1/2 activation

The Src family kinases have been implicated as important mediators of ERK1/2 activation through GPCRs and RTKs (45, 46, 47). The mechanisms for some of the proposed signal transduction sequences have been considererd in the Introduction (chapters, 2.3.1.1, 2.3.1.2 and 2.3.2.3). PP2 is a potent inhibitor of the Src family tyrosine kinases and selectively inhibits Lck, Fyn and Hck. The non-functional peptide PP3 was added as a negative control for the inhibitor PP2. PP2 interfered strongly with ERK1/2 activation in our system in a concentration dependent manner, whereas PP3 had no effect at 20 µM (fig. 4.12A and B).

The tyrosine residue 416 in the C-terminal lobe on Src becomes phosphorylated upon activation. Treatment with 5-HT did not change the degree of phosphorylation of this residue compared to the basal phosphorylation observed in KB1 cells (fig. 4.13) or in HEK293 cells transiently transfected with 5-$HT_{7(b)}$ receptors (data not shown).

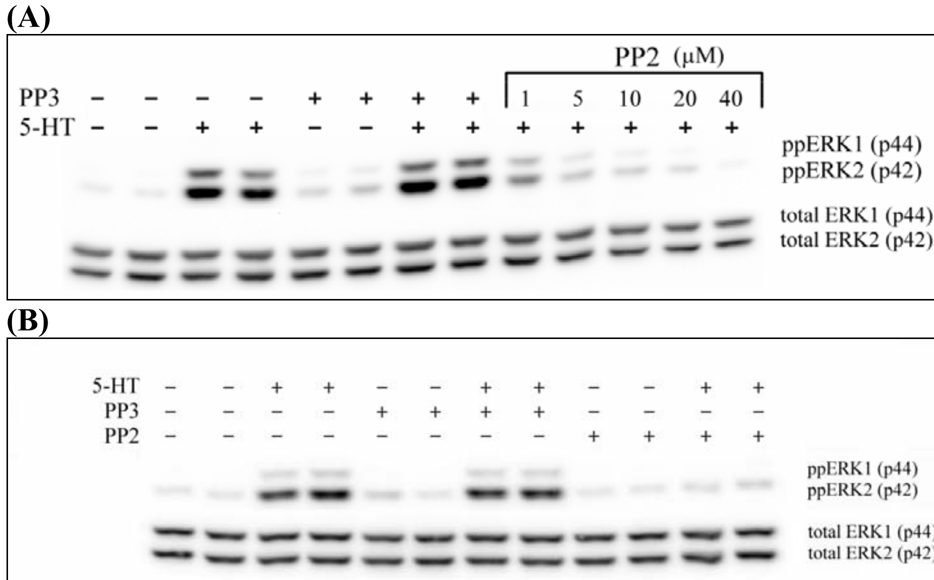


Figure 4.12 The Src inhibitor PP2 interferes with the activation of ERK1/2. **A,** KB1 cells stably expressing 5-$HT_{7(b)}$ receptors were pretreated with 20 μM PP3 for 25 min, or various concentrations of PP2 as indicated for 25 minutes, prior to treatment with 5-HT (10 μM) or vehicle for 5 minutes. **B,** KB1 cells were pretreated with PP3 (20 μM) or PP2 (20 μM) as indicated for 25 minutes, prior to treatment with 5-HT (10 μM) or vehicle for 5 minutes. The cells in were harvested and processed as described in fig. 4.1. Panel **B** shows a representative blot of three experiments, two of which in KB1 cells and one in transiently transfected cells (5-$HT_{7(b)}$ receptors).

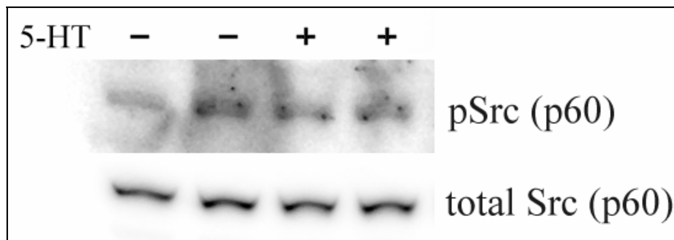


Figure 4.13 Phosphorylation of Src in KB1 cells. KB1 cells stably expressing 5-$HT_{7(b)}$ receptors were with 5-HT (10 μM) or vehicle for 5 minutes as indicated. The cells were harvested and separated as described in fig. 4.1. The western blot was probed with phosphospecific Src family antibodies (p*Src*; *upper panel*) and subsequently probed with anti-Src antibodies (total Src; *lower panel*) to confirm equal loading.

4.1.8 The role of Ca^{2+} in ERK1/2 activation

Thapsigargin is a drug that inhibits the Ca^{2+}-ATPase family of calcium pumps on sarcoplasmic or endoplasmic reticulum, thus mediating a slow but enduring elevation of cytoplasmic calcium levels (71). An increase in cytoplasmic calcium levels mediated by thapsigargin leads to activation of ERK1/2 in many cell systems (72). As shown in figure 4.14, ERK1/2 is activated in KB1 cells treated with thapsigargin, indicating that increased levels of cytoplasmic calcium is sufficient to induce a phosphorylation of ERK1/2 in this cell system.

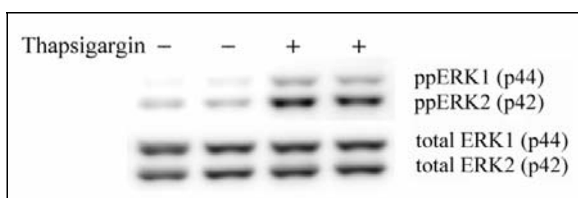


Figure 4.14 Activation of ERK1/2 is induced by thapsigargin. KB1 cells stably expressing 5-$HT_{7(b)}$ receptors were pretreated with thapsigargin (1 μM) for 5 min. The cells were harvested and processed as described in fig. 4.1. The figure shows a representative blot of three similar experiments.

Furthermore, the calcium chelator BAPTA-AM was used to evaluate the role of calcium. BAPTA-AM is able to cross plasma membranes in its AM (acetoxymethyl) ester form only. The AM form is inactive, but BAPTA-AM is activated by the cleavage of AM by intracellular esterases. The phosphorylation of ERK1/2 was reduced in the presence of BAPTA-AM (fig. 4.15), indicating that intracellular Ca^{2+} plays a role in the activation of ERK1/2 mediated through 5-$HT_{7(b)}$ receptors.

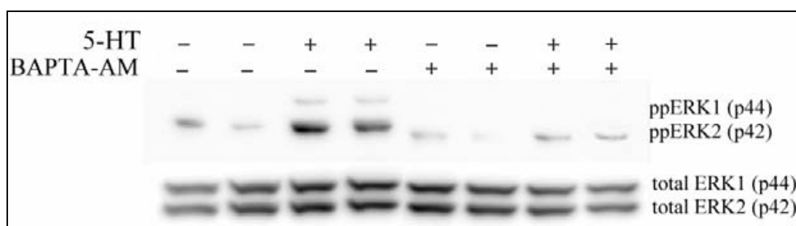


Figure 4.15 The calcium chelator BAPTA-AM interferes with the phosphorylation of ERK1/2. KB1 cells stably expressing 5-$HT_{7(b)}$ receptors were pretreated with BAPTA-AM (40 μM) for 25 minutes prior to treated with 5-HT (10 μM) for 5 minutes. The cells were harvested and processed as described in fig. 4.1. The figure shows a representative blot of four experiments, two in KB1 cells and two in HEK293 cells transiently transfected with 5-$HT_{7(b)}$ receptors.

The calcium inhibitor CAI, which inhibits receptor mediated and voltage gated calcium influx non-specifically (73,74), was used to further assess the role of Ca^{2+} in the activation of ERK1/2 mediated through 5-$HT_{7(b)}$ receptors. The use of CAI in the KB1 cell system reduced activation of ERK1/2 substantially (fig. 4.16).

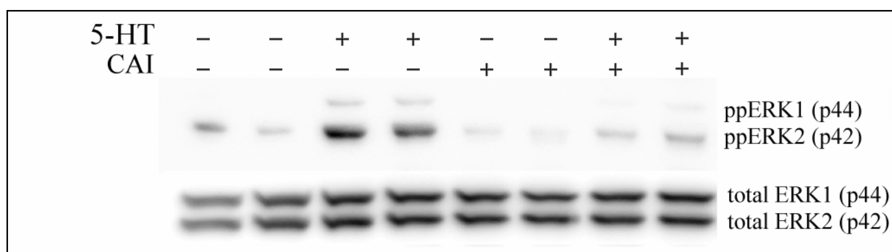


Figure 4.16 Activation of ERK1/2 is blocked by calcium channel inhibitor CAI. KB1 cells stably expressing 5-$HT_{7(b)}$ receptors were pretreated with CAI (20μM) for 25 minutes prior to treatment with 5-HT (10 μM) for 5 minutes. The cells were harvested and processed as described in fig. 4.1. The figure shows a representative blot of six experiments, four of which in KB1 cells and two in HEK293 cells transiently transfected with 5-$HT_{7(b)}$ receptors.

Gadolinium (element 64) blocks calcium channels in its Gd^{3+} form. Gd^{3+} does not readily cross plasma membranes due to its strong hydrophilic nature and thus exerts its effects on plasma membrane calcium channels mainly.

The effects of Gd^{3+} on the observed 5-HT induced activation of ERK1/2 are not consistent. In three experiments, the presence of Gd^{3+} interfered with ERK1/2 activation (fig. 4.17A) but in four experiments there was no such interferance (fig. 4.17B). The actual effect of Gd^{3+} on the observed 5-HT induced activation of ERK1/2 is still not certain.

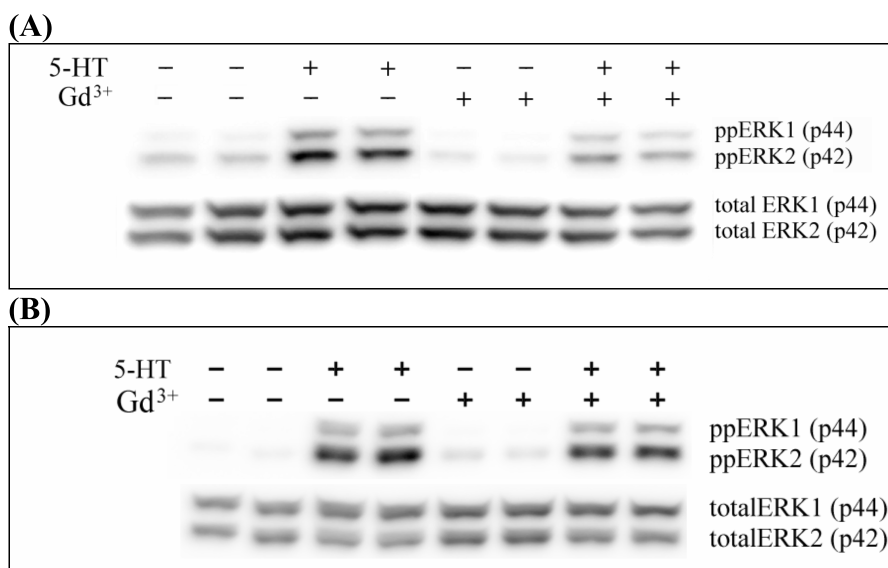


Figure 4.17 Variable effects of a plasma membrane calcium channel inhibitor. A and **B,** KB1 cells stably expressing 5-$HT_{7(b)}$ receptors were pretreated with $GdCl_3$ (20μM) for 25 minutes prior to treatment with 5-HT (10 μM) for 5 minutes. The cells were harvested and processed as described in fig. 4.1. Panel **A** shows a representative blot of four experiments where there was an effect of $GdCl_3$, three performed in KB1 cells and one in HEK293 cells transiently transfected with 5-$HT_{7(b)}$ receptors. Panel **B** shows a representative blot of three experiments where there was no effect of $GdCl_3$, two in KB1 cells and one in HEK293 cells transiently transfected with 5-$HT_{7(b)}$ receptors.

To investigate the specificity and effect of the calcium channel blockers Gd^{3+} and CAI more thoroughly, experiments with thapsigargin and the blockers were conducted. Gd^{3+} did not inhibit thapsigargin-induced ERK1/2 activation (fig. 4.18), in fact it augmented ERK1/2 activation. CAI, which is known to cross plasma membranes, interfered strongly with thapsigargin-induced ERK1/2 activation (fig. 4.18), indicating that CAI is able to block calcium channels effectively.

Experiments were also conducted with EGF and the calcium channel blockers to see if they interfered with EGF-induced ERK1/2 activation. Neither Gd^{3+}, nor CAI, inhibited EGF-induced ERK1/2 activation significantly (fig. 4.19). In two experiments however, EGF-induced ERK1/2 activation was augmented significantly in the presence of Gd^{3+} (data not shown).

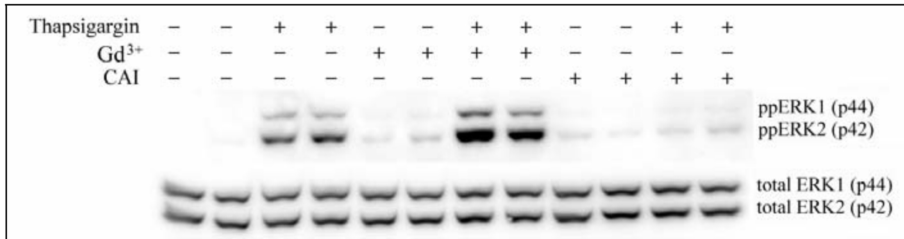


Figure 4.18 Effects of Gd^{3+} and CAI on thapsigargin-induced phosphorylation of ERK1/2. HEK293 cells transiently transfected with 5-$HT_{7(b)}$ receptors were pretreated with $GdCl_3$ (20 μM) or CAI (20 μM) for 25 minutes prior to treatment with thapsigargin (1 μM) for 5 minutes as indicated. The cells were harvested and processed as described in fig. 4.1. The figure shows a representative blot of two similar experiments.

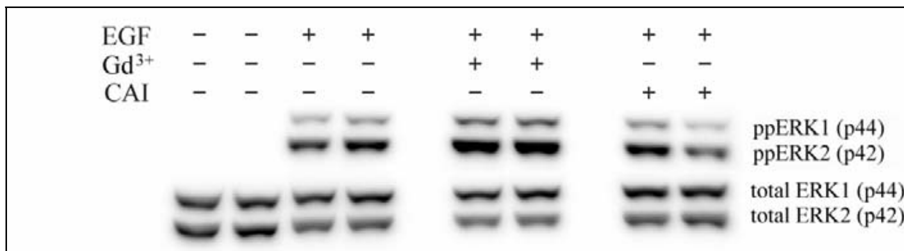


Figure 4.19 EGF induced ERK1/2 activation is not inhibited by Gd^{3+} or CAI. HEK293 cells transiently transfected with 5-$HT_{7(b)}$ receptors were pretreated with $GdCl_3$ (20 μM) or CAI (20 μM) for 25 minutes prior to treatment with EGF (10 nM) for 5 minutes as indicated. The cells were harvested and processed as described in fig. 4.1. The figure shows a representative blot of three experiments, one in KB1 cells and two in HEK293 cells transiently transfected with 5-$HT_{7(b)}$ receptors.

4.1.9 The role of Ras-GRF1 in ERK1/2 activation

The role of the Ca^{2+}/calmodulin-activated Ras-specific exchange factor Ras-GRF1 (see chapter 2.3.1.4 for details) was investigated. The activity of Ras-GRF1 towards Ras is enhanced by PKA-dependent phosphorylation of Ser898, which corresponds to the mouse Ser916 site. The two sites share identical amino acid sequences, and thus Ser916 antibodies for the mouse sequence of Ras-GRF1 can be used to detect the human Ser898 site. Human Ras-GRF1 is mainly expressed in neurons of the central nervous system, and to a certain extent in the pancreas and lungs.

HEK293 cells were cotransfected with plasmids encoding the 5-$HT_{7(b)}$ receptors and plasmids encoding HA-tagged Ras-GRF1-wild-type (HA-Ras-GRF1-wt). Overexpression of HA-Ras-GRF1-wt resulted in a massive elevation of the basal level of phosphorylation of ERK1/2 which was increased further subsequent to 5-HT stimulation (fig. 4.20).

We found that Ras-GRF1 is expressed endogenously in HEK293 cells, and that it becomes phosphorylated on Ser898 subsequent to 5-HT stimulation in KB1 cells and HEK293 cells transiently transfected with 5-$HT_{7(b)}$ receptor (fig. 4.21A). The same is

observed in HEK293 cells cotransfected with 5-$HT_{7(b)}$ receptors and HA-Ras-GRF1-wt (fig. 4.21B). As can be seen from figure 4.21B, HA-Ras-GRF1-wt is massively overexpressed when cotransfected into HEK293 cells.

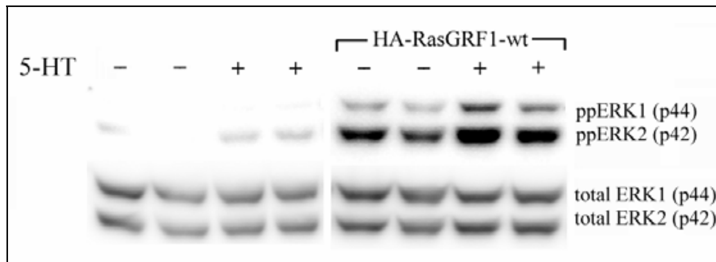
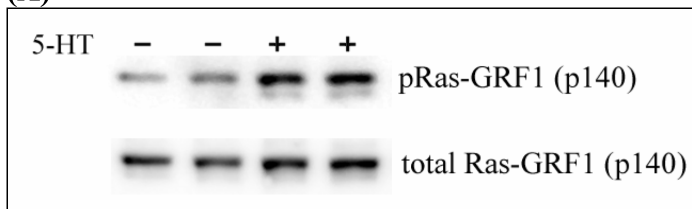


Figure 4.20 Ras-GRF1-wt activates ERK1/2. HEK293 cells transiently cotransfected with plasmids encoding the 5-$HT_{7(b)}$ receptor and plasmids encoding HA-Ras-GRF1-wt, were treated with 5-HT (10 μM) for 5 minutes as indicated. The cells were harvested and processed as described in fig. 4.1. The figure shows a representative blot of three similar experiments.

(A)



(B)

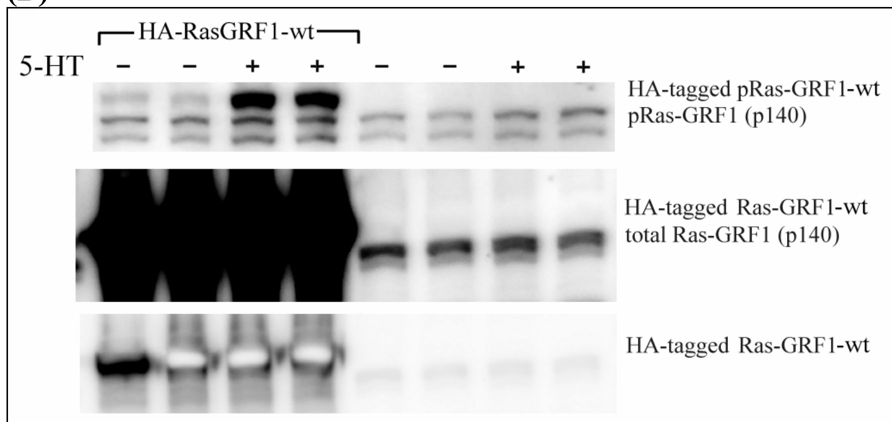


Figure 4.21 Ras-GRF1 is present in HEK293 cells and becomes phosphorylated on Ser898. **A,** HEK293 cells transiently transfected with plasmids encoding the 5-$HT_{7(b)}$ receptor, were treated with 5-HT (10 μM) for 5 minutes. **B,** HEK293 cells were cotransfected with 5-$HT_{7(b)}$ receptors and HA-Ras-GRF1-wt and treated with 5-HT (10 μM) for 5 min as indicated. The cells were harvested and separated as described in fig. 4.1. The Western blots were probed with Ser916 phosphospecific Ras-GRF1 antibodies (*pRas-GRF1*; *A and B top panels*) and subsequently probed with anti-Ras-GRF1 antibodies (*total Ras-GRF1*; *A and B middle and bottom panels*) to confirm equal loading. The *middle panel* in **B** was exposed for 5 minutes, whereas the *bottom panel* was exposed for 20 seconds. Panel **A** shows a representative blot of nine experiments, four in HEK293 cells transiently transfected with 5-$HT_{7(b)}$ receptors, and five in KB1 cells. Panel **B** shows a representative blot of two similar experiments.

The Ser898 residue is a PKA specific phosphorylation site, and since PKA is dependent on cAMP and 5-$HT_{7(b)}$ receptors couple to $G\alpha_s$, we wanted to investigate whether a reduction in cAMP would interfere with the phosphorylation of this site.

HEK293 cells were cotransfected with 5-$HT_{7(b)}$ receptors and hPDE4D2 (described in chapter 4.1.3). 5-HT-induced phosphorylation of Ser898 was abolished in the presence of overexpressed $hPDE_{4D2}$, which suggests that this phosphorylation is indeed dependent on cAMP (fig. 4.22).

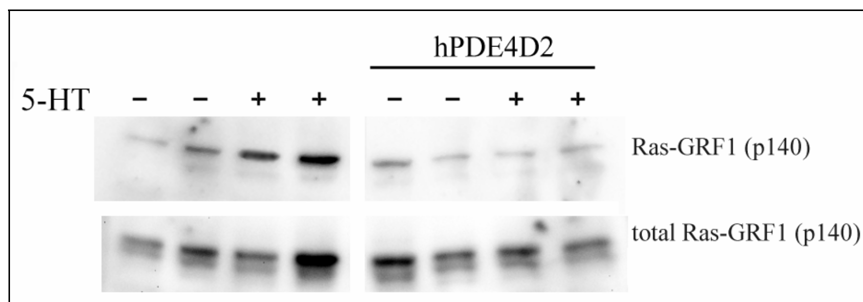


Figure 4.22 Phosphorylation of Ras-GRF1 on Ser898 is dependent on cAMP. HEK293 cells transiently cotransfected with plasmids encoding the 5-$HT_{7(b)}$ receptor and $hPDE_{4D2}$ were treated with 5-HT (10 μM) for 5 minutes. The cells were harvested and processed as described in fig. 4.21B. The figure shows a representative blot of three similar experiments.

The PKA inhibitor H89 has been used with various results in previous experiments (figure 4.7A and B), and the effect of this drug as an inhibitor of phosphorylation on the PKA specific site Ser898 on Ras-GRF1 is unclear. Interestingly, in the experiments where H89 has no effect on ERK1/2 activation (fig. 4.7A), there is little effect on Ras-GRF1 phosphorylation (fig. 4.23A), and vice versa (fig. 4.7B and 4.23B), indicating that PKA is indeed necessary for both ERK1/2 activation and Ras-GRF1 phosphorylation, and that the variable effects of H89 on ERK1/2 activation could be due to a variable efficiency of H89 to block PKA.

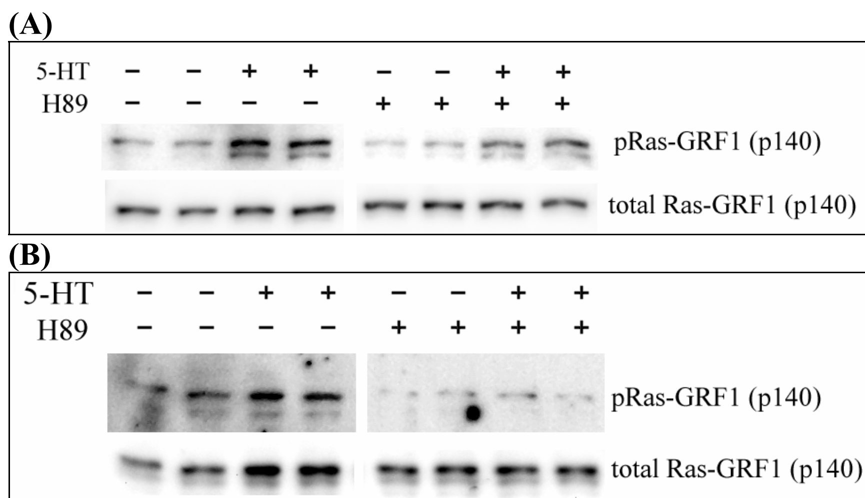


Figure 4.23 H89 interferes with the phosphorylation of Ras-GRF1. **A** and **B**, HEK293 cells transiently transfected with plasmids encoding the 5-$HT_{7(b)}$ receptor were pretreated with H89 (20 μM) for 30 minutes and treated with 5-HT (10 μM) for 5 minutes. The samples are the same as described in fig. 4.7. The cells were harvested and processed as described in fig. 4.21B. Panels **A** and **B** each show representative blots of two experiments.

The role of PKA in the phosphorylation of Ser898 on Ras-GRF1 was further evaluated by cotransfection of HEK293 cells with 5-$HT_{7(b)}$ receptors and PKI (described in chapter 4.1.4). As previously experienced, the effectiveness of PKI as an inhibitor of PKA is somewhat variable. In fact, the situation is the same as for H89, as described earlier; In the experiments where PKI blocked phosphorylation of Ras-GRF1 (fig. 4.24B) it also blocked activation of ERK1/2 (fig. 4.8B), and vice versa (fig. 4.23A and 4.8A), again indicating that PKA is in fact necessary for both ERK1/2 activation and Ras-GRF1 phosphorylation.

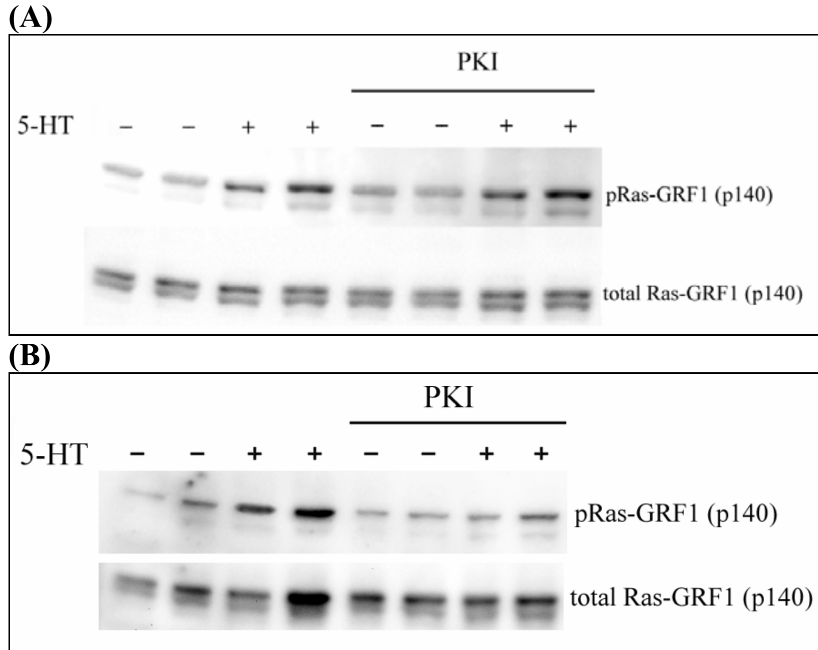


Figure 4.24 PKI interferes with the phosphorylation of Ras-GRF1. A and **B**, HEK293 cells transiently cotransfected with plasmids encoding the 5-$HT_{7(b)}$ receptor and PKI were treated with 5-HT (10 μM) for 5 minutes. The samples are the same as described in fig. 4.8. The cells were harvested and processed as described in figure 4.21B. Panels **A** and **B** each show representative blots of two experiments.

EGF strongly activates ERK1/2 through a mechanism known to be independent of Ras-GRF1, To determine the specificity of the observed phosphorylation of Ras-GRF1 induced by 5-HT, HEK293 cells transiently transfected with 5-$HT_{7(b)}$ receptors were treated with EGF. As shown in figure 4.25, treatment with EGF did not result in phosphorylation of Ras-GRF1 on Ser898.

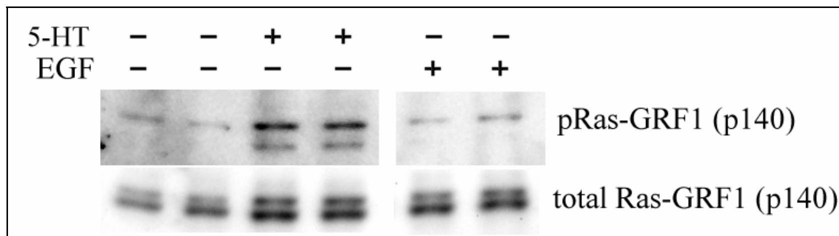


Figure 4.25 Phosphorylation of Ras-GRF1 is not induced by EGF. HEK293 cells transiently cotransfected with plasmids encoding the 5-$HT_{7(b)}$ receptor were treated with 5-HT (10 μM) for 5 minutes. The cells were harvested and processed as described in fig. 4.21B. The figure shows a representative blot of two identical experiments.

4.1.10 The role of Calmodulin and CaMKII in ERK1/2 activation

Calmodulin is a very important effector of calcium signals, and CaMKII is in turn an effector of calmodulin. CaMKII has been implicated in the activation of ERK1/2 (75). More importantly, Ca^{2+}/calmodulin is the main activator of Ras-GRF1 (34). We therefore wanted to assess whether these proteins play a part in ERK1/2 activation mediated through 5-$HT_{7(b)}$ receptors. However, neither the calmodulin inhibitor W-7 (fig. 4.25A), nor the CaMKII inhibitor KN-93 (fig. 4.25B) interfered with ERK1/2 activation in our system.

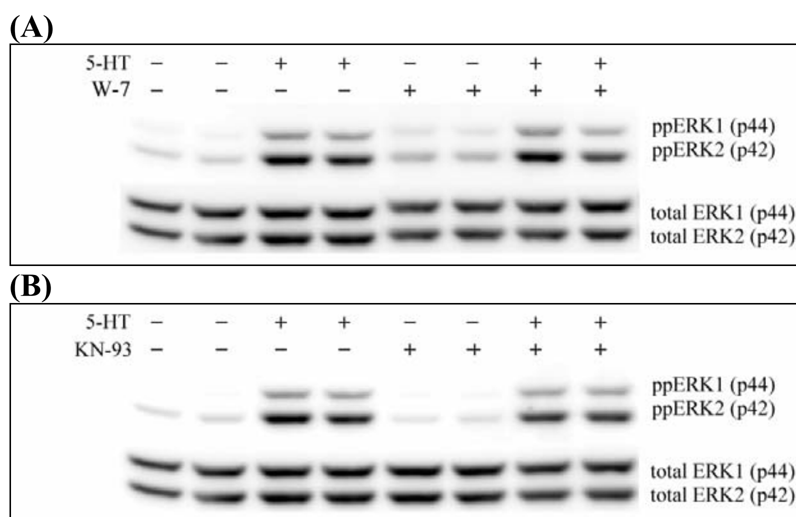


Figure 4.26 Activation of ERK1/2 is not inhibited by a calmodulin blocker (A) or a CaMKII blocker (B). A and **B,** KBI cells stably expressing 5-$HT_{7(b)}$ receptors were pretreated with **A**, W-7 (25 µM) or **B**, KN-93 (20 µM) for 25 minutes prior to 5 min treatment with 5-HT (10 µM) as indicated. The cells were harvested and processed as described in fig. 4.1. Panels **A** and **B** each show representative blots of three experiments, for each of these three experiments, one was conducted in HEK293 cells transiently transfected with 5-$HT_{7(b)}$ receptors and two in KBI cells.

4.2 Direct Cytosolic Calcium Measurements

Based on the results obtained with the various calcium inhibitors, there were reasons to believe that cytosolic calcium levels were elevated subsequent to stimulation of the 5-$HT_{7(b)}$ receptors with serotonin, and that the observed ERK1/2 activation could be dependent on such an increase. This possibility was further explored by direct cytosolic calcium measurements with fura-2, which make it possible to measure variations in cytosolic calcium concentrations in real time. Not all cells generate elevations in cytosolic calcium after stimulation, and only responders were selected for further studies. In our experiments about 60-80% of the KBI cells produced a detectable calcium signal after stimulation with serotonin, which is regarded as normal (prof. J.G. Iversen, personal communication).

4.2.1 The 5-$HT_{7(b)}$ receptors mediate elevation of intracellular Ca^{2+}

Our data show that there is a rapid but transient elevation of free intracellular Ca^{2+} levels of 60-70% over basal after stimulation with serotonin in the KBI cells (fig. 4.27). In single cells we observed increases of up to 130% over basal (data not shown). The duration of the main peak varied somewhat between experiments, with a rapid but transient elevation lasting about 30-40 s as shown in fig. 4.27, and more elongated plateaus lasting for up to 60 s (data not shown).

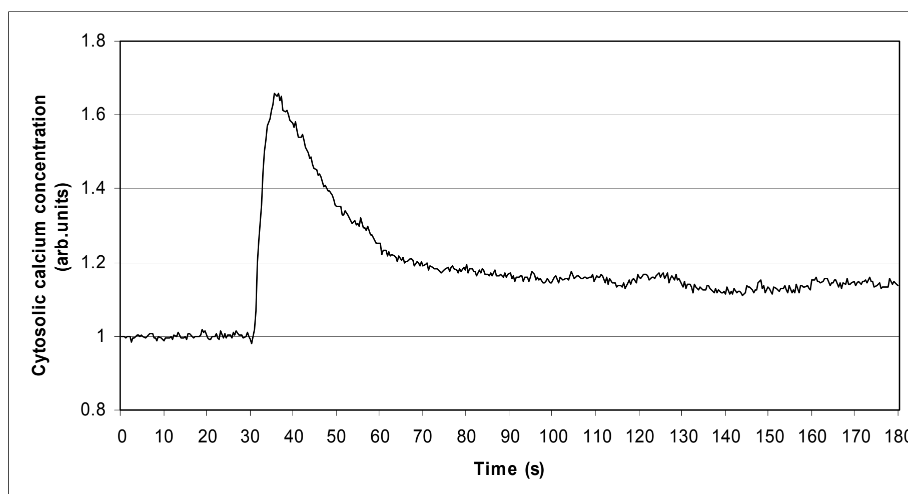


Figure 4.27 The levels of free cytosolic calcium is elevated in KBI cells subsequent to 5-HT stimulation. KBI cells stably expressing 5-$HT_{7(b)}$ receptors were loaded with fura-2 for 20 min at room temperature, and direct cytosolic calcium measurements were carried out as described under Methods. The cells were treated with 5-HT (10 μM) 30 seconds after the start of the recordings. The graph is composed of the average cytosolic calcium concentrations in 15 single cells in one experiment. The figure is a representative graph of 18 similar experiments.

4.2.2 Thapsigargin induces elevation of intracellular Ca^{2+}

The KBI cells were treated with thapsigargin as a positive control for elevation of free intracellular calcium in this cell system. Treatment with thapsigargin induces a slow but long-lasting increase in cytosolic calcium levels. The rise in calcium commences about 30 s after stimulation, and a peak level of about 50% over basal is seen after 60 s (fig. 4.28).

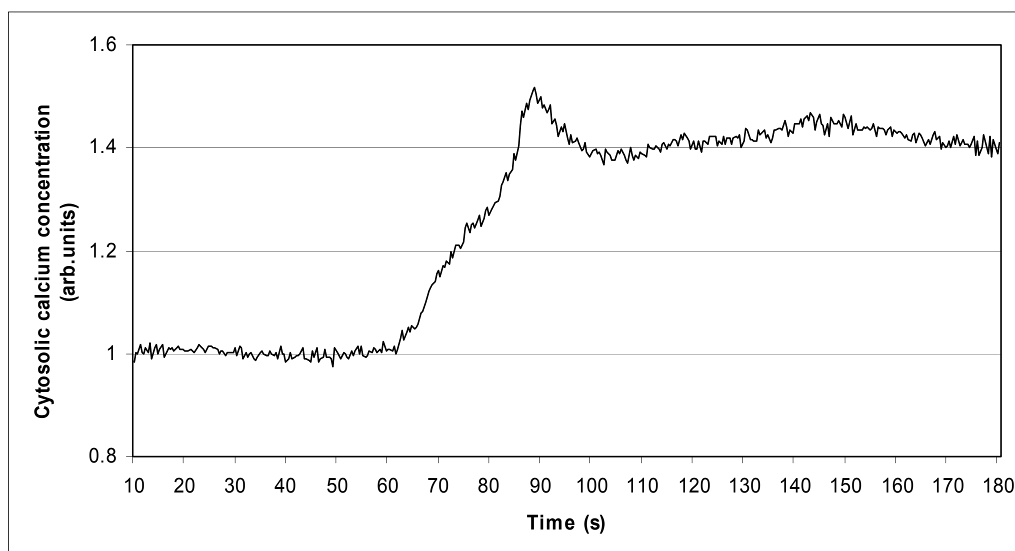


Figure 4.28 The levels of free cytosolic calcium is elevated in KBI cells subsequent treatment with thapsigargin. KBI cells stably expressing 5-$HT_{7(b)}$ receptors were loaded with fura-2 for 20 min at room temperature, and direct cytosolic calcium measurements were carried out as described under Methods. The cells were treated with thapsigargin (1 μM) 30 seconds after the start of the recordings. The graph is composed of the average cytosolic calcium concentrations in 17 single cells in one experiment. The figure is a representative graph of three similar experiments.

4.2.2 The role of PKA in the elevation of intracellular Ca^{2+}

PKA has been shown to phosphorylate and thus activate ryanodine receptors and voltage-gated calcium channels. Based on previous data we wanted to determine the role of PKA in the 5-HT induced increase in free intracellular calcium in HEK293 cells. As shown in fig. 4.29, the previously observed elevation in cytosolic calcium concentrations wa completely abolished when the cells were preincubated with the PKA inhibitor H89. However, H89 also eliminated the thapsigargin-induced increase in free cytosolic calcium (fig. 4.30). To our knowledge there is no published data supporting a role for PKA in the thapsigargin induced increase in free cytosolic calcium.

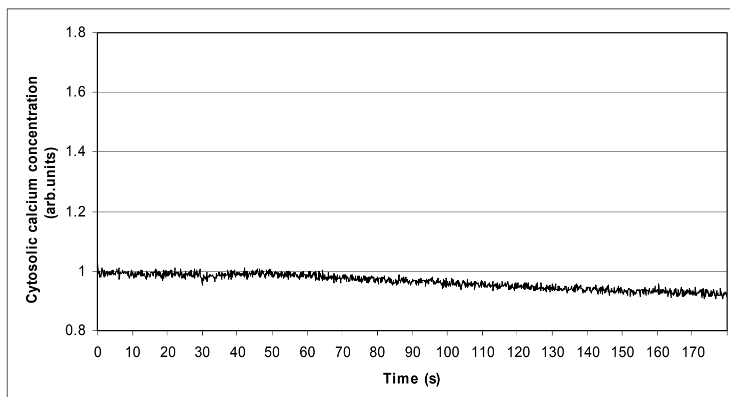


Figure 4.29 5-HT-induced elevation of free cytosolic calcium is abolished in the presence of H89. KBI cells stably expressing 5-$HT_{7(b)}$ receptors were loaded with fura-2 for 20 min at room temperature, and direct cytosolic calcium measurements were carried out as described under Methods. The cells were pretreated with H89 (20 µM) for 20 minutes. 5-HT (10 µM) was added after 30 seconds in the presence of 20 µM H89. The graph is composed of the average free cytosolic calcium concentrations in 25 single cells in one experiment. The figure is a representative graph of six similar experiments.

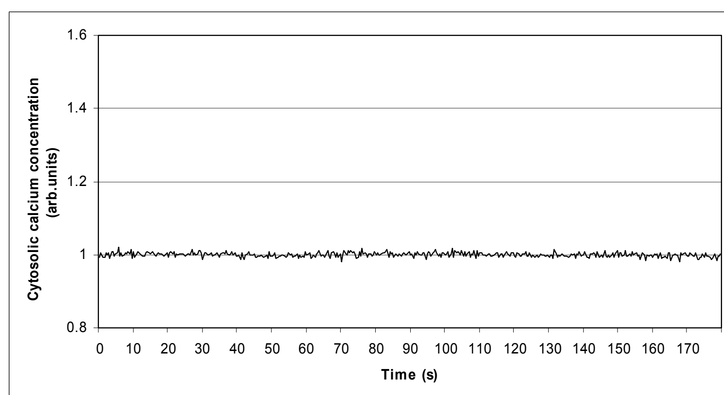


Figure 4.30 Thapsigargin-induced elevation of cytosolic calcium is abolished in the presence of H89. KBI cells stably expressing 5-$HT_{7(b)}$ receptors were loaded with fura-2 for 20 min at room temperature, and direct cytosolic calcium measurements were carried out as described under Methods. The cells were pretreated with H89 (20 µM) for 20 minutes. Thapsigargin (1 µM) was added after 30 seconds in the presence of 20 µM H89. The graph is composed of the average cytosolic calcium concentrations in 30 single cells in one experiment. The figure is a representative graph of three similar experiments.

5 Discussion

5.1 Experimental Considerations

5.1.1 Methodological limitations

When evaluating the results presented in this thesis, it is important to bear in mind that the methods used to obtain these results have certain limitations. Each band on a Western blot represents proteins from the cells of one 35 mm well dish, and there are many handling steps involved before such bands are visualized. The quality of the cells and drugs, the variability in transfection rates, gel electrophoresis, transfer of proteins by Western blotting and attachment of primary and secondary antibodies to the membranes, are all factors that contribute to the reliability of the final outcome. It is reasonable to conclude that our results must be interpreted mainly as semi-quantitative, and the data must be assessed with caution. The bands on the membranes are evaluated visually in relation to the other bands on the same membrane, and in essence this consists in determining whether the bands have lower, similar or higher intensities. As expected, this makes it difficult to detect subtle changes, and bands on different membranes may generally not be compared to each other. It is possible to get a "quantitative" evaluation of the immunoassayed membranes by optical densitometric scanning, but as can be expected from the intrinsic variability in the results produced by our methods, this is not a very proficient way to assess the data, and may even produce artificial results. It is difficult, if not impossible to determine how to limit the scanned bands, and air bubbles, background noise and the appearance of the bands on a membrane may interfere significantly with the final numerical outcome. As such, optical densitometric scannings have not been conducted systematically in this thesis.

If not otherwise stated, most of the experiments have been conducted in duplicates and repeated at least three times.

5.1.2 Receptor densities on the KB1 cells

The KB1 cells is a clone of HEK293 cells stably transfected with 5-$HT_{7(b)}$ receptors and was made in our lab previously (courtesy of dr. K.A. Krobert). They exhibit fairly high receptor levels, 25000 fmol/mg protein, which may affect the data obtained with these cells. This is discussed further in chapter 5.2.

5.1.3 Transfection

Transfection is used to introduce plasmids into eukaryotic cells. Transfection rates are however, never 100%, and as a consequence, some cells remain without the desired plasmid, and thus do not express the protein it encodes. For example, in the case of cotransfection with dominant negative RasN17 and 5-HT receptors, the nontransfectecd cells may exhibit some activity of Ras, and thereby induce a basal phosphorylation of ERK1/2 which interferes with the final result. This basal activity is not, however, induced by stimulation with serotonin. On the other hand, there will always be a pool of endogenous proteins left, even in transfected cells. So although RasN17 disrupts signaling from Ras specific exchange factors to Raf-1, endogenous Ras may still functions normally and mediate phosphorylation of ERK1/2.

5.2 The Experiments – Activation of ERK1/2

In this thesis, we report 5-HT-induced activation of ERK1/2 mediated through the human G_s-coupled serotonin receptors 5-$HT_{7(b)}$ and 5-$HT_{7(d)}$ transiently expressed in HEK293 cells, and for the 5-$HT_{7(b)}$ receptors, in stably transfected HEK293 cells, the KB1 clone.

Most of the work was done with the 5-$HT_{7(b)}$ receptor and is part of an ongoing project in our lab where the human G_s-coupled serotonin receptors, 5-HT_7 and 5-HT_4 are investigated concurrently. Unless otherwise stated, the following discussion relates to the activation of ERK1/2 through 5-$HT_{7(b)}$ receptors in HEK293 cells.

The main propagators of intracellular signals subsequent to stimulation of G-protein-coupled receptors, are the Gα- and Gβγ-subunits of heterotrimeric G proteins. $G\alpha_s$ couples to and stimulates AC with subsequent elevation of cAMP. Cotransfection with hPDE4D2 and 5-$HT_{7(b)}$ receptors almost completely abolishes ERK1/2 activation and demonstrates that this activation is largely dependent on cAMP.

Except for some ion channels, the two main targets for cAMP are PKA and Epac. Both PKA and Epac have been proposed to play a role in the activation of Rap1. However, Rap1 is mainly activated through the Rap1 specific exchange factor Epac. We determined the role of Rap1 in the observed 5-HT-induced activation of ERK1/2 by cotransfecting HEK293 cells with the Rap1 specific GTPase activating protein Rap1GAP1 and 5-HT receptors. The observed 5-HT-induced activation of ERK1/2 in HEK293 cells was not influenced by co-expression of Rap1GAP1. In addition, stimulation with the Epac-specific cAMP analogue 8CPT-2Me-cAMP had no effect on ERK1/2 activation. Thus, in HEK293 cells the G_s-coupled 5-$HT_{7(b)}$ receptors mediate phosphorylation of ERK1/2 through a Rap1-independent pathway, in contrast to the pathway reported for β_2-ARs (21): G_s-AC-cAMP-PKA-Rap1-B-Raf-MEK-ERK.

PKA has been shown to inhibit RTK-mediated activation of ERK1/2 in a cell type specific manner, and phosphorylation of Raf-1 is the main target for PKA-mediated regulation of the ERK1/2 cascade (45). Pretreatment of transiently transfected HEK293 cells with H89 only sporadically and partially suppressed 5-HT-induced dual phosphorylation of ERK1/2. Cotransfection with the PKA inhibiting peptide PKI also produced similarly erratic results. However, the data considering the phosphorylation of Ras-GRF1 shed some light on this discrepancy. Residue Ser898 on Ras-GRF1 is a PKA-specific phosphorylation site. It is anticipated that inhibition of PKA will interfere with this phosphorylation. In the experiments where H89 failed to interfere with activation of ERK1/2, it also failed to block phosphorylation on Ser898, and vice versa; in the experiments where H89 had an effect, both the phosphorylation of Ras-GRF1 and ERK1/2 was inhibited. This indicates that PKA may play a role, but questions the effectiveness of H89 and the used PKI construct as inhibitors of PKA. In addition, the data obtained from direct cytosolic calcium measurements were also bewildering since H89 completely abolished thapsigargin-induced elevation of cytosolic calcium. To our knowledge there is no known role for PKA in thapsigargin induced elevation of free intracellular Ca^{2+} levels, and therefore, this discredits H89 further as a “selective, potent inhibitor of cAMP-dependent protein kinase” as stated by the manufacturer (76). Davies et al. (77) has reported that H89 is not a selective PKA inhibitor, which is in agreement with our findings that H89 must be used with caution. We have hypothesized that combined treatment with fura-2 and H89 is too harsh on the cells, and merely kills them. This may explain the

dramatic effects of H89 on 5-HT- and thapsigargin-induced calcium elevation, and we are currently undertaking experiments to verify whether this is the case.

Currently we can not conclude from the data presented whether the 5-HT-induced phosphorylation of ERK1/2 through 5-$HT_{7(b)}$ receptors is mediated via a PKA-dependent pathway, but there are indications that activation of ERK1/2 through 5-$HT_{7(b)}$ receptors is at least partly dependent on PKA.

An alternative pathway could have been mediated through the PI3-kinase, which has been shown to be activated by free Gβγ-subunits and to be a factor in the activation of ERK1/2 in some cell systems. However, the PI3K inhibitor wortmannin did not interfere with ERK1/2 activation. These data suggest that PI3K does not play a role in the phosphorylation of ERK1/2 through 5-$HT_{7(b)}$ receptors.

As for the 5-$HT_{7(a)}$ receptors, ERK1/2 activation through 5-$HT_{7(b)}$ and 5-$HT_{7(d)}$ receptors is dependent on the small G-protein Ras. The presence of the constitutively active mutant of Ras, RasV12 activated ERK1/2 strongly, whereas the dominant negative mutant of Ras, RasN17 almost completely abolished phosphorylation of ERK1/2. Phosphorylated ERK1/2 levels were not, however, reduced entirely to basal levels with RasN17 and a possible explanation for this is discussed in chapter 5.1.3. Of course, it is also possible that other pathways are in action and constitute the remaining activation of ERK1/2.

Our main focus has been to elucidate how Ras becomes activated, and the majority of the work in this thesis has been dedicated to uncover a possible signal transduction mechanism leading from elevated levels of cAMP to activated GTP-bound Ras.

Several studies have shown that the stimulation of 5-HT_7 receptors leads to an increase in cytosolic calcium levels (65,67). Furthermore, calcium has been proposed to play an essential role in the activation of ERK1/2 for many receptor systems. We demonstrate that the concentration of free intracellular calcium is increased rapidly and transiently in KB1 cells after stimulation with serotonin. This result, however, is somewhat dubious as it may be a result of receptor overexpression as mentioned in chapter 5.2.1. We know that the 5-$HT_{7(b)}$ receptor density is very high in the KB1 cells, and it has been hypothesized that such a large receptor number may cause unspecific effects (78), *e.g.* coupling to other G proteins. This theory is supported by the appearance of the calcium influx curve which resembles that of a G_q coupled receptor (see chapter 5.1.2).

We further show that thapsigargin induces an elevation of intracellular calcium and mediates activation of ERK1/2. The calcium chelator BAPTA-AM interferes with ERK1/2 activation mediated through 5-$HT_{7(b)}$ receptors, indicating that the availability of intracellular calcium is necessary for the proper activation of ERK1/2. The calcium channel blocker CAI also interfered with the observed ERK1/2 activation, indicating that functional calcium channels are required. The plasma membrane calcium channel blocker Gd^{3+} only interfered sporadically with ERK1/2 activation. We can only speculate over the reasons for this. One possibility could be that gadolinium ions have gained access to the cytoplasm of the cell in some experiments, and have thereby inhibited the intracellular calcium channels necessary for proper ERK1/2 activation. We found that Gd^{3+}, in contrast to CAI, actually augmented thapsigargin-induced, as well as EGF-induced and basal non-stimulated ERK1/2 activation in several experiments. This may suggest that Gd^{3+}, when restricted from access to intracellular calcium channels, actually prevents leakage of calcium from the

cell by blocking the plasma calcium channels, thus augmenting Ca^{2+}-induced ERK1/2 activation. CAI, which is known to cross plasma membranes, interfered strongly with thapsigargin-induced ERK1/2 activation but did not interfere with EGF-induced ERK1/2 activation. These data indicate that CAI is able to block calcium channels without interfering with the classical Ca^{2+}-independent RTK-Ras-Raf-MEK pathway.

Our data show that: i) ERK1/2 is activated by increased levels of intracellular calcium; ii) Stimulation of 5-$HT_{7(b)}$ receptors in HEK293 cells leads to increased levels of intracellular calcium; and iii) Treatment with either of two calcium inhibitors (BAPTA-AM and CAI) interferes with ERK1/2 activation through 5-$HT_{7(b)}$ receptors.

Taken together we propose that the observed activation of ERK1/2 through 5-$HT_{7(b)}$ receptors is mediated via a calcium-dependent pathway. We have not been able to determine from which reservoirs the observed increase in free intracellular calcium levels originates (intracellular/extracellular reservoirs, or a combination). Neither do we know how the influx of calcium is brought about. We have speculated that PKA may be involved, but the lack of molecular tools has so far hindered us from further investigation of this intriguing prospect. Pretreatment with H89 in the direct cytosolic calcium measurements caused a complete abolishment of calcium elevation subsequent to 5-HT stimulation, but as mentioned previously, these results are futile as H89 also eradicated thapsigargin-induced calcium elevation.

The nature of calcium signal transduction mechanisms is a very complex area of research. There are many receptor systems and regulators involved, and even the presence of the most specific inhibitors may result in numerous non-specific cellular effects as can be anticipated when interfering with such a crucial and fine-tuned second messenger.

Ca^{2+}/calmodulin activates the Ras specific exchange factor Ras-GRF1, which could be one of several exchange factors mediating activation of Ras subsequent to stimulation of 5-HT_7 receptors. We demonstrate that: i) Ras-GRF1 is expressed endogenously in HEK293 cells; ii) Ras-GRF1 is phosphorylated on the PKA specific site Ser898 following stimulation of 5-$HT_{7(b)}$ receptors in HEK293 cells; iii) ERK1/2 is activated constitutively in HEK293 cells cotransfected with HA-Ras-GRF1-wt and 5-$HT_{7(b)}$ receptors, and this ERK1/2 activation is augmented even further after stimulation with 5-HT.

Calcium is a necessary component for the activation of Ras-GRF1 through binding of Ca^{2+}/calmodulin. We have demonstrated that intracellular calcium is elevated in KB1 cells subsequent to stimulation with 5-HT, but we don't know whether this calcium alone is sufficient to activate ERK1/2. The observed increase in free intracellular calcium is within an order of magnitude as that of thapsigargin, but the thapsigargin-response lasts much longer, which makes a direct comparison difficult.

We have not investigated the kinetics in these reactions, but the calcium levels peaked and declined rapidly. The onset was immediate and lasted from 30-60 seconds subsequent to 5-HT stimulation. However, activation of ERK1/2 peaked after 4-7.5 minutes. It can be envisioned that some of the calcium becomes bound to calmodulin after influx, and that Ca^{2+}/CaM binds to and activates Ras-GRF1. However, the calmodulin antagonist W-7 failed to inhibit ERK1/2 activation in our system. This may indicate that Ca^{2+}/calmodulin is not necessary for the observed activation of ERK1/2. However, we have not obtained data confirming a positive effect of W-7 in the HEK293 cells. Experiments to confirm the activity of W-7 are currently being undertaken in our laboratory. If this finding is authentic, however, it weakens the Ras-GRF1-hypothesis considerably, but does not exclude a role for

Ras-GRF1, especially since we have demonstrated that over-expressed HA-Ras-GRF1-wt mediates activation of ERK1/2 and that this activation is augmented after 5-HT stimulation.

The CaM kinase II is another effector of calmodulin thought to be able to mediate activation of ERK1/2 through many mechanisms, *e.g.* phosphorylation and activation of Raf-1 (75). However, KN-93, an inhibitor of CaMKII, did not interfere with ERK1/2 activation suggesting that CaMKII does not play a role in ERK1/2 activation through 5-$HT_{7(b)}$ receptors.

Just as for PKA and Ca^{2+}, the cell biology concerning calmodulin and CaMKII is very complex and involves a plethora of signal transduction mechanisms and localizations of substrates. A simplified example may illustrate this point: Apart from activating Ras-GRF1, Ca^{2+}/Calmodulin also activates PDE1 (79) which lessens activation of ERK1/2 by reducing intracellular levels of cAMP. It is thus possible to envision that inhibition of calmodulin produces both negative and positive effects on ERK1/2 activation much the same way as PKA does, and this adds considerably to the complexity when investigating such signal transduction mechanisms. This is especially true in a situation where many separate pathways contribute to the final activation of ERK1/2, which might well be the case in our cell system. Taken together, the use of an inhibitor may result in an increase, decrease or no significant change in the activation of ERK1/2, depending on how essential the opposing mechanisms are. Of course, it is possible to construct such ad hoc propositions for all the data presented in this thesis if one were so inclined, and the point is that further evidence must be presented before final conclusions can be drawn.

Several possible mediators of ERK1/2 activation were studied further. Especially interesting are the Src family kinases, which have been implicated in the activation of ERK1/2 through G_s-coupled receptors. Klinger et al. reported that elevation of cAMP in many cell systems caused an ERK1/2 phosphorylation which was inhibited by PP1, a Src kinase inhibitor (46). They did not report any data concerning HEK293 cells, but we found that the Src kinase inhibitor PP2 almost completely abolishes phosphorylation of ERK1/2 through 5-$HT_{7(b)}$ receptors whereas the negative control (the inactive analog PP3) had no effect. This indicates that Src kinases play an important role in ERK1/2 activation.

The residue Tyr416 on Src is autophosphorylated when the Src kinases are activated, and can thus be used as a marker of Src activation. We have detected a basal level of Tyr416-phosphorylated Src, but no increased phosphorylation on this conserved residue subsequent to stimulation of 5-$HT_{7(b)}$ receptors in HEK293 cells. Consequently, we do not have indications that Src kinases are activated in our system after treatment with 5-HT, but we have not investigated the kinetics of these reactions, and it could be that Src autophosphorylation of Tyr416 is a transient event which is difficult to detect. The cell biology of the Src kinases, as every other component of a cell, is subject to many control mechanisms. The Src family kinases may be phosphorylated on different residues, producing activation or inhibition. For example, it has also been proposed that PKA can phosphorylate and activate Csk, which in turn can phosphorylate and thereby inhibit Src family kinases (80). Our data show that inhibition of the Src family kinases reduces the activation of ERK1/2 through 5-$HT_{7(b)}$ receptors, but as of yet, we have not conducted any further experiments to clarify exactly how the Src family kinases contribute to ERK1/2 activation. One can only speculate whether Src plays a direct or indirect role. It could be

that Src kinases are necessary for the overall metabolic apparatus in a cell, and disrupting them may thus decrease activation of ERK1/2 non-specifically. Needless to say, such a prospect needs further assessment.

5.3 Future Research

The role of PKA should be assessed further. Due to the fact that the available inhibitors exhibit several weaknesses as discussed in previous chapters, other strategies should be employed to disrupt PKA signaling. The use of siRNA-technology is promising in this regard and has been used with success to knock down PKA expression completely (19).

It is also of interest to explore further the role of calcium on ERK1/2 activation and the exact mechanism for how the 5-HT_7 receptors mediate increase in free intracellular calcium. Direct cytosolic calcium experiments should be conducted with cell lines expressing lower receptor densities to verify the data from the KB1 clone. Various calcium inhibitors can be used to shed more light on how increased levels of intracellular calcium are brought about, for example L-type and T-type channel blockers (*e.g.* verapamil and mibefradil respectively) to determine whether voltage gated calcium channels contribute to calcium influx in HEK293 cells. It will be important to find out to what extent ryanodine receptors are involved in calcium influx, and inhibitors (ryanodine, procaine) and inducers (caffeine) of RyRs can be employed to shed more light on this. Moreover, the roles of calmoduline, CaM-kinases and Src family kinases must be studied further.

It is still unknown if and to what extent Ras-GRF1 contributes to the activation of Ras in our system. We have started screening for functional siRNA constructs to knock down Ras-GRF1 expression to see whether this interferes with ERK1/2 activation.

5.4 Conclusions

In conclusion, we demonstrate activation of ERK1/2 induced by 5-HT through the human G_s-coupled serotonin receptors 5-$HT_{7(b)}$ and 5-$HT_{7(d)}$ in HEK293 cells. The 5-$HT_{7(b)}$ receptor has been chosen as a model for further studies and the observed phosphorylation of ERK1/2 is cAMP- and Ras-dependent but Epac- and Rap1-independent. We show that stimulation of 5-$HT_{7(b)}$ receptors lead to a rapid but transient increase in intracellular calcium through an unknown mechanism, and that the observed activation of ERK1/2 is at least partly dependent on this increase in free intracellular calcium. We demonstrate that Ras-GRF1 is present endogenously in HEK293 cells and that it is phosphorylated on Ser898 subsequent to stimulation of 5-$HT_{7(b)}$ receptors, but its complete role in ERK1/2 activation is still unknown. The role of the main effector of cAMP, PKA, remains elusive, but there are indications that activation of ERK1/2 through 5-$HT_{7(b)}$ receptors is at least partly dependent on PKA.

Figure 5.1 gives a schematic representation of the possible signal transduction mechanisms leading from stimulation of the 5-$HT_{7(b)}$ receptors to the activation of ERK1/2 in HEK293 cells.

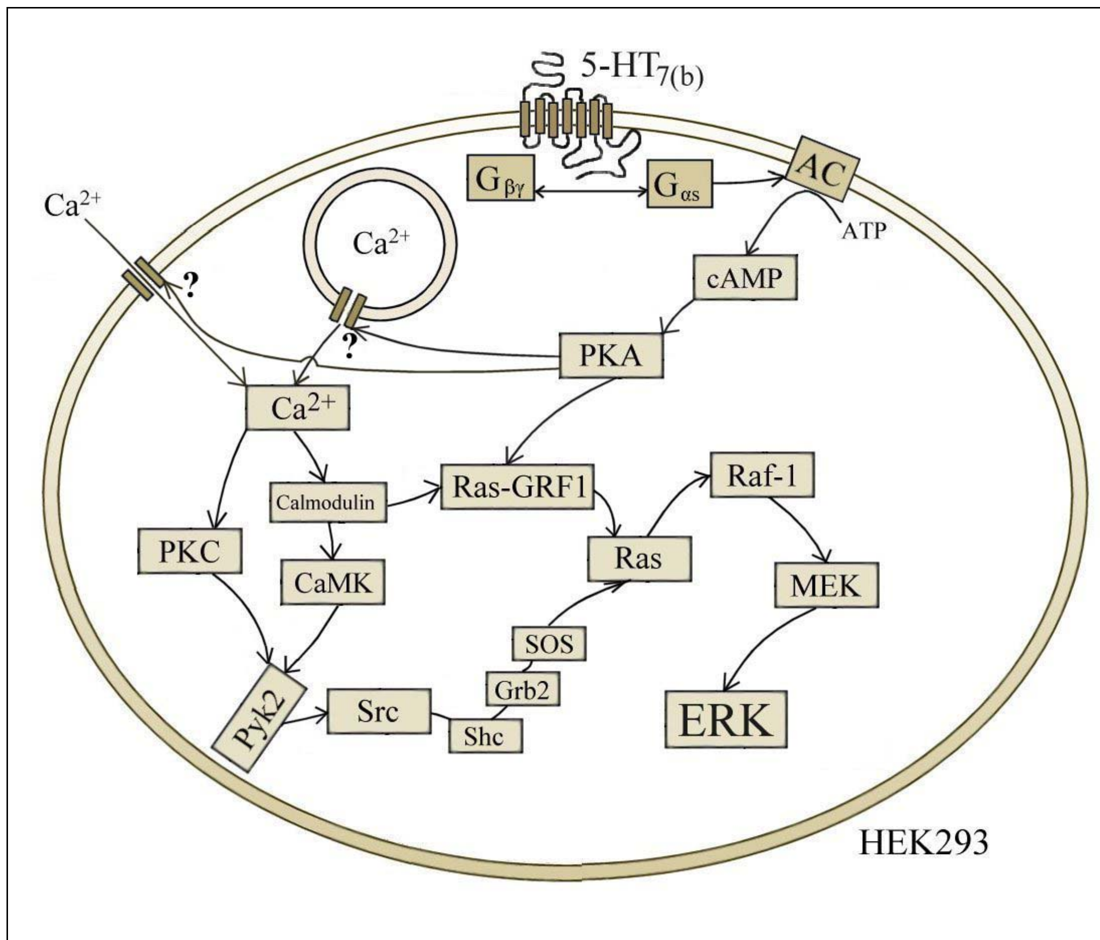


Figure 5.1 Possible molecular signal transduction pathways leading from the 5-$HT_{7(b)}$ receptor to activation of ERK1/2 in HEK293 cells.
Question marks indicate areas of uncertainty that need further clarification.

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7 Appendix

7.1 Abbreviations

Abbreviation	*Full name*
β-AR	β-adrenergic receptor
5-HT	5-hydroxytryptamine (serotonin)
AC	adenylyl cyclase
ADP	adenosine-5'-diphosphate
AGS	activator of G protein signaling
AKAP	A kinase anchor protein
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
CalDAG-GEF	calcium- and diacylglycerol regulated GEF
CaM/CaMK	calmodulin/calmodulin kinase
cAMP	adenosine-3',5'-cyclic monophosphate
cGMP	guanosine-3',5'-cyclic monophosphate
CNS	central nervous system
CTx	cholera toxin
CREB	cAMP response element-binding protein
DAG	1,2-diacylglycerol
DNA	deoxyribonucelic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Epac	exchange protein directly activated by cAMP
ERK	extracellular signal-regulated kinase
G-protein	guanine nucleotide-binding protein
GAP	GTPase activating protein
GDP	guanosine-5'-diphosphate
GEF	guanine exchange factor
GPCR	G protein-coupled receptor
Grb2	growth-factor receptor bound protein 2
GRK	G protein-coupled receptor kinase
GTP	guanosine-5'-triphosphate
HEK293	human embryonic kidney 293 cell
IP_3	inositol triphosphate
Jak	Janus tyrosine kinase
MAPK	mitogen activated protein kinase
MEK	MAP and ERK kinase
PDZ	PSD–95 (postsynaptic density), Dgl (Discs–large), and ZO (zonula occludans)
PI3K	phosphoinositide 3-kinase
PKA	Protein kinase A (cAMP-dependent protein kinase)
PKC	Protein kinase C (Ca^{2+}-dependent protein kinase)
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PTx	pertussis toxin
Pyk2	Proline-rich tyrosine kinase 2

Rap1	Ras analogue protein 1
Ras	rat sarcoma
RasGRF	Ras guanine nucleotide releasing factor
RasGRP	Ras guanine nucleotide releasing protein
RGS	regulator of G protein signaling
RNA	ribonucleic acid
RT	room temperature (22ºC)
RTK	receptor tyrosine kinase
SH2/SH3	Src homology 2 or 3 domain
Shc	SH2 domain containing
SOS	son of sevenless
Src	Sarcoma (v-Src – viral Src, c-Src – cellular (mammalian) Src)
STAT	signal transducers and activators of transcription
TM	transmembrane

7.2 Materials and Recipes

7.2.1 Chemicals and antibodies

Chemical	***Abbreviation***	***Manufacturer***
1-butanol, graded for analysis, min 99,5%	butanol	Merck
5-hydroxytryptamine hydrochloride (serotonin)	5-HT	Sigma
Acrylamide 4K, ultrapure	acrylamide	AppliChem
Agarose	agarose	Gibco
Ammonium Persulfate	APS	Bio-Rad
Ampicillin, sodium salt, min 98%	ampicillin	Sigma
Bacto-Agar	agar	Difco
Bacto-Tryptone	tryptone	Difco
Bacto-Yeast extract	yeast extract	Difco
BAPTA-AM	BAPTA-AM	Calbiochem
BCA Protein Assay Reagent	BCA-kit	Pierce
Bisacrylamide 4K, ultrapure	bisacrylamide	AppliChem
Cryoprotective medium	Cryoprotective medium	BioWhittaker
dimethylsulphoxide, sterile filtered	DMSO	Sigma
Dulbecco’s Modified Eagles Medium, 4.5 g glucose/L	DMEM	Sigma
Ethanol	ethanol	Arcus
Epidermal growth factor	EGF	Sigma
Ethidium bromide	ethidium bromide	Sigma
Fetal bovine serum	FBS	Gibco
Fura-2-AM	Fura-2-AM	TefLabs
Gadolinium (III) chloride hexahydrate 99%	gadolinium	Sigma
Glycin 99.8%	glycin	AppliChem
H89 (N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride)	H89	Sigma
Hank’s balanced salt solution, 1x w/phenol red	HBSS	BioWhittaker
Hydrocholoric acid 37%, p.a.	HCl	Merck
KN-93 (2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine))	KN-93	Calbiochem

L-651582 (carboxyamidotriazole)	CAI	McKesson
Lipofectamine 2000	lipofectamine 200	Invitrogen
Lipofectamine reagent	lipofectamine	Invitrogen
Methanol, p.a.	methanol	PROLABO
N',N',N',N'-tetra-metyl-ethylene-diamide	TMED	AppliChem
Non-fat dry milk	dry milk	Nestle
Optimem	optimem	Gibco
Phosphate buffered saline, 10x powder, ultrapure grade	PBS	Amresco
Pluronic acid	pluronic acid	Sigma
PP2 (4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine)	PP2	Calbiochem
PP3 (4-Amino-7-phenylpyrazol[3,4-d]pyrimidine)	PP3	Calbiochem
QIAGEN Plasmid Maxi Kit	QIAGEN maxiprep	QIAGEN
SOC-medium	SOC-medium	Gibco
Sodium Chloride, min 99%	NaCl	Sigma
Sodium dodecyl sulphate, ultrapure, min 99.5%	SDS	Gibco
Sodium hydroxide	NaOH	Merck
Sodium-ortho-vanadate	Na_3VO_4	Sigma
SuperSignal West Dura Extended Duration Substrate	SuperSignal	Pierce
Thapsigargin	thapsigargin	Calbiochem
TOP-10 Chemically competent E.coli cells	TOP-10 cells	Invitrogen
Trizma base min 99.9% (Tris[hydroxymethyl]aminomethane)	tris base	Sigma
Trizma hydrochloride min 99% (Tris[hydroxymethyl]aminomethane hydrochloride)	tris-HCl	Sigma
Trypsin/Versene EDTA 0.5g/L	trypsin	BioWhittaker
Tween-20	tween	Bio-Rad
Ultraculture general purpose serum free medium	Ultraculture	BioWhittaker
W-7 (N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride)	W-7	Sigma
Wortmannin	wortmannin	Sigma
β-mercaptoethanol, min 98%	β-mercaptoethanol	Sigma

Antibody	***Species***	***Manufacturer/prod.number***
Anti-phospho-p44/42 MAP Kinase	Mouse	Cell signaling (#9106L)
Anti-MAP-kinase 1/2 (ERK 1/2-CT)	Rabbit	Upstate biotecnology (06-182)
Anti-mouse IgG HRP linked	Sheep	Amersham biosciences (NXA931)
Anti-rabbit IgG HRP linked7	Donkey	Amersham biosciences (NA934)
Anti-phospho-Ras-GRF1 (Ser916)	Rabbit	Cell signaling (#3321)
Anti-Ras-GRF1 (c-20)	Rabbit	Santa Cruz biotechnology (sc-224)
Anti-phospho-Src (Tyr416)	Rabbit	Cell signaling (#2101)
Anti-Src	Mouse	Oncogene (#OP07)

7.2.1.1 Contents of commercial kits

QIAGEN Plasmid Maxi Kit

Buffer P1 (Resuspension) 50 mM Tris-Cl, pH 8.0 10 mM EDTA 100 µg/ml RNase A	**Buffer QBT (Equilibration)** 750 mM NaCl 50 mM MOPS pH 7.0 15% isopropanol
Buffer P2 (Lysis) 200 mM NaOH 1% SDS	**Buffer QC (Wash)** 1.0 M NaCl 50 mM MOPS pH 7.0 15% isopropanol
Buffer P3 (Neutralization) 3.0 M potassium acetate pH 5.5	**Buffer QF (Elution)** 1.25 M NaCl 50 mM Tris-Cl pH 8.5 15% isopropanol

7.2.2 Solutions and buffers

7.2.2.1 Amplification of DNA plasmids

TE Buffer 10 mM Tris-Cl pH 8.0 1 mM EDTA	**LB Medium** 10 g tryptone 5 g yeast extract 10 g NaCl ad 1000 ml H_20 pH 7.0 Autoclave

7.2.2.2 Direct cytosolic calcium measurements

Hepes buffer 10 mM Hepes 1.2 mM Ca^{2+} pH 7.37 288 mOsm	**Fura-2-AM Loading Buffer** 50 µg Fura-2-AM (50 nmol) is thawed for 30 minutes protected from light and dissolved in 30 µl DMSO/Pluronic acid (10 % solution), and kept dark for 30 minutes. The Fura-2-AM stock solution is diluted in 10 ml Hepes buffer (5 µM Fura-2-AM)

7.2.2.3 SDS-PAGE and Western blotting

Harvest solution 35 mM SDS 1 mM Na_3VO_4 50 mM Tris pH 7.4	
1.5 M Tris-base pH = 8.8 181.71 g Tris-base 900 ml dH20 Adjust pH to 8.8 with conc HCl at RT ad 1000 ml dH20 Stored at RT	**1.0 M Tris-base pH = 6.8** 60.57 g Tris-base 400 ml dH20 Adjust pH to 6.8 with conc HCl at RT. ad 500 ml dH20 Stored at RT
30% Acrylamidemix 4.08 M Acrylamide 64.86 mM N,N'-methylenebisacrylamide stored in dark bottle up to 4 weeks at RT	**4x Loading Buffer (for loading samples)** 12.5 ml 1.0 M Tris-HCl pH = 6.8 40 ml 10% SDS 40 ml glycerol 99% 0.4 ml bromophenol blue ad 100 ml dH20 Activate with 10% β-mercaptoethanol Stored at RT.
10 ml 10% polyacrylamide gel (ERK1/2 assay) 4 ml dH20 3.3 ml 30% acrylamide 2.5 ml 1.5 M Tris-base pH 8.8 100 µl 10% SDS 100 µl 10% APS (fresh) 4 µl TEMED	**5x Running buffer (for SDS-PAGE)** 124.65 mM Tris-base 1.24 M g glycine 17.34 mM SDS Stored at RT
10 ml 6% polyacrylamide gel (Ras-GRF1 assay) 5,3 ml dH20 2 ml 30% acrylamide 2.5 ml 1.5 M Tris-base pH 8.8 100 µl 10% SDS 100 µl 10% APS (fresh) 8 µl TEMED	**10x Transfer buffer (for Western blotting)** 312.86 mM Tris-base 2.4 M glycine Stored at RT
3 ml 5% polyacrylamide gel (stacking gel) 2 ml dH20 0.5 ml 30% acrylamide 0.375 ml 1.0 M Tris-base pH 6.8 30 µl 10% SDS 30 µl 10% APS (fresh) 2 µl TEMED	**700 ml blotting buffer (for each Western blotting chamber)** 70 ml 10x Transfer buffer 490 ml dH20 140 ml methanol Stored at 4ºC